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Development and characterisation of novel antimicrobial phosphate glasses for urinary tract infections.

Farah Naz Safdar Raja

Doctor of Philosophy

Aston University

September 2017

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Thesis summary

Urinary tract infections (UTIs) affect significant proportions of the population, particularly prevalent in elderly patients. Up to 40 % of healthcare associated infections are UTIs and 80% of those are associated with catheter use. CA-UTIs significantly effect patient's health and are frequently associated with substantial morbidity and mortality. Furthermore, the financial implications are enormous as the high complication rate arising from catheterisation requires significant time and cost. In the UK, more than a million cases are reported each year accounting for annual economic burden of approximately £125 million. The aim of this study was to develop novel antimicrobial bioactive glasses that can be used to prevent and treat catheter associated urinary tract infections. This was achieved by assessing the antimicrobial efficacy of increasing cobalt or zinc content (1, 3, 5 and 10 mol %) in phosphate based glass system (P_2O_5 - Na_2O - CaO). Glass compositions with two metal oxides (cobalt, copper or zinc) were also studied to determine synergistic combinations against a panel of clinically relevant microorganisms. A decrease in the dissolution rates of cobalt glasses was seen with increasing cobalt content, however an increase in dissolution rate was observed with higher zinc content (5% and 10%). A strong antimicrobial activity was exhibited by 5 and 10 mol % cobalt or zinc doped glasses which was not only time dependent but also strain specific. Moreover, combinations such as Co/Cu, Co/Zn and Cu/Zn showed synergism against *E. coli* and *S. aureus*. Whilst a strong antimicrobial activity was seen, the cytotoxic studies demonstrated decrease in cell viability of mammalian cells when exposed to glasses directly and their dissolution products. Nevertheless, cobalt and/or zinc doped phosphate based glasses could potentially be used as a cartridge in drainage bags or to coat catheters with a decreasing amount to prevent cytotoxic effects.

Keywords: Phosphate based glasses, antibacterial, cytotoxicity, cobalt and zinc.

To my beloved father

I wish I can wipe 24th July 2017 from calendar, I wish you lived a little longer, I wish I met you one last time and hugged you and kissed you, I wish I never said to you I can't talk for long I am writing my thesis, I wish I phoned you every day.

To the world you were a sincere, honest and straightforward person, your charismatic personality and kind nature earned you the utmost respect in society. For us you were the most friendly, loving and caring father, I do not have words to express my feelings, you were simply the best. I never realised the true value of the moments I spent with you until they became a memory. I miss you.

*The fact that you are no longer with us, causes immense pain but you are forever in our hearts
Until we meet again, InshaAllah'*

May Allah grant you the highest place in Jannah' Ameen.

This is for you Dad!

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List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometer
ANOVA	Analysis of Variance
Ag	Silver
ATCC	American Type Culture Collection
Ca ²⁺	Calcium ion
CaCO ₃	Calcium carbonate
CA-UTIs	Catheter Associated Urinary Tract Infections
CFU	Colony Forming Units
Ch	Charrierers
cm ²	Centimetre Squared
Co	Cobalt
CO ₂	Carbon dioxide
CRG	Controlled Release Glass
Cu	Copper
CVC	Central Venous Catheter
D/E	Dey/Engley
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
EPS	Exopolysaccharide

FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Indices
FBS	Foetal Bovine Serum
g	Gram
HAI	Healthcare Associated Infection
HEXRD	High Energy X-ray Diffraction
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
ISO	International Standardization Organisation
LPS	Lipopolysacchrides
MBC	Minimum Bactericidal Concentration
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
mL	Millilitre
mm	Millimetre
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrients Agar
NB	Nutrients Broth
NCTC	National Collection of Type Cultures
NF	Nitrofurazone
OD	Optical Density
P	Phosphorus
PBS	Phosphate Buffered Saline
P/S	Penicillin/Streptomycin
PVC	Polyvinyl chloride

PTFE	Polytetrafluoroethylene
ROS	Reactive Oxygen Species
rpm	Resolutions Per Minute
SBF	Simulated Body Fluid
SEM	Scanning Electron Micrograph
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
T/E	Trypsin/EDTA
TNS	Trypsin Neutralising Solution
UCM	Urothelial Cell Media
UCGS	Urothelial Cell Growth Supplement
UTI	Urinary Tract Infection
v/v	Volume per Volume
w/v	Weight per Volume
XRD	X-Ray Diffraction
Zn	Zinc

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Chapter 1

Introduction and Aims

1.1 Overview

In most developed countries, as the life expectancy has increased there is an increased demand on the healthcare system. Medical devices are an integral part of modern medicine. Whilst this has been a huge success in improving the healthcare facilities, the occurrence and undesirable complications arising from such devices have well been recognized and continue to escalate [1]. Since the presence of a foreign body creates a favourable environment for microbial colonisation, device related infections therefore account for at least half of the healthcare associated infection [2]. One of the most commonly reported device related infection is catheter associated urinary tract infection (CA-UTI) due to the inability of the material to prevent microbial colonisation [3, 4].

Urinary catheters have long been used in hospitals and nursing homes. A number of patients who are admitted in hospital require urinary catheterisation at some stage during their stay. Urinary tract infections (UTIs) are the most common type of hospital - acquired infection accounting for up to 40% of all cases and 80% of those are associated with catheter use [5]. There are various reasons for catheterisation ranging from collecting a sterile urine sample to treating a long term underlying conditions such as urinary incontinence due to bladder weakness or nerve damage. In the latter case an indwelling catheter is inserted into the urinary tract via the urethra for an extended period of time thus predisposing the patient to urinary tract infection [3]. Since the catheters are made of different polymers, they provide favourable conditions for the growth and attachment of microorganisms thus leading to biofilm formation. These infections are difficult to treat as antibiotics fail to penetrate biofilms and thus can not only cause considerable morbidity but also can prove fatal. Therefore, due to the severity and the complications associated with catheter related urinary tract infections prevention of catheter related infections is an area that requires further investigation.

The aim of this chapter is to provide a brief overview of human urinary system, the need for urinary catheterisation and associated complications, mainly the infection, caused by it. The causative agents and their route of entry, pathogenesis of the infection will also be discussed in detail. Prevention of these infection by use of antimicrobial catheters, associated limitations and the concept of using bio-glasses as a potential alternative will be explored.

1.2 Urinary System

1.2.1 Components and Functions

The urinary system is one of the major systems in the body that serves to maintain haemostatic balance of the body. It consists of two kidneys, two ureters, the bladder and urethra (Figure 1.1). Kidneys receive plasma portion of the blood and remove urea (nitrogenous waste product) from the body through millions of nephrons. Kidneys also play a central role in regulating water concentration, maintaining correct ionic composition (potassium, sodium, calcium, magnesium). The required components get selectively reabsorbed back into the blood to maintain proper balance whilst waste products that do not get filtered become secreted into the urine [6]. Other important functions include production of hormones and release of erythropoietin, which controls red blood cell production in the bone marrow.

Urine produced by the kidneys is pushed down through ureters into urinary bladder by peristaltic movements. **Ureters** are narrow, muscular, thick walled tubes which are 25-30 cm in length and 3-4 mm in diameter [6]. Smooth muscles in ureter walls continuously tighten and relax to avoid building up of urine in kidneys. Small amounts of urine are emptied into the bladder every 15-20 seconds [7]. The ureters empty into **urinary bladder**, a triangular-shaped, hollow organ located in the lower abdomen. It serves to store urine until it is convenient to release it. The typical healthy adult bladder, on average can store up to 300-400 ml of urine before emptying however can hold

up 700 ml [8]. Three different types of muscles control the voiding of urine from bladder; internal sphincters, external sphincters and pelvic floor muscles that provide additional support to bladder [8]. When the bladder is full, stretch muscles send signals to parasympathetic nervous system and urination or micturition happens by voluntary relaxation of external sphincters muscles leading to opening of internal sphincters. Finally, during urination, urine is discharged from the body via **urethra**. Due to the innate mechanism of the human body, any bacteria (normally the endogenous species that colonize the periurethral area) that ascend the urinary tract are flushed out during micturition.

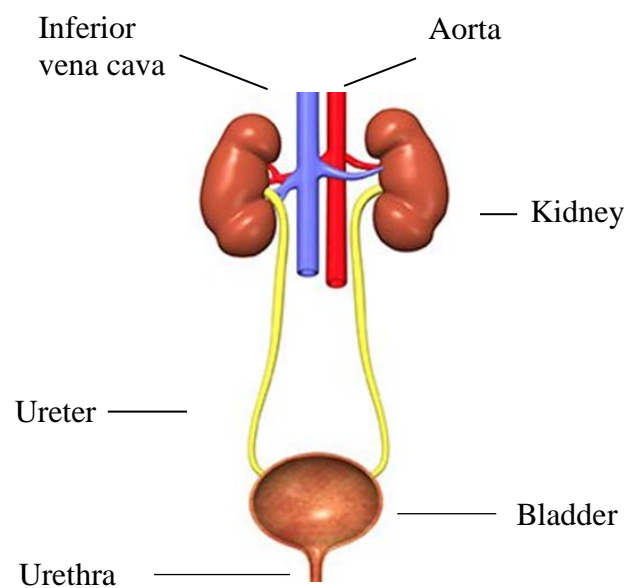


Figure 1.1: The human urinary tract system [9].

1.2.2 The Human Urothelium

The urinary bladder like renal pelvis, ureters and upper urethra is lined by a tissue called uroepithelium or urothelium (Figure 1.2), which forms an interface between urinary space and the underlying tissues [10]. It is a transitional epithelium composed of three layers; basal, intermediate and superficial layer. The urothelium responds to stretch via large hexagonal cells present in the outermost layer called umbrella cells. In addition, these cells are coated with glycosaminoglycan mucin that act as the first line of defence against microbial invasion by preventing the adherence of bacteria [10, 11].

When the bladder starts to fill up, the cells in the outer layer begin to change their shape and can elongate ten times their original size, enabling the bladder to store urine. Other than allowing urine storage, urothelium serves as a barrier between the outer environment (excretory products i.e. urine) and the underlying tissues.

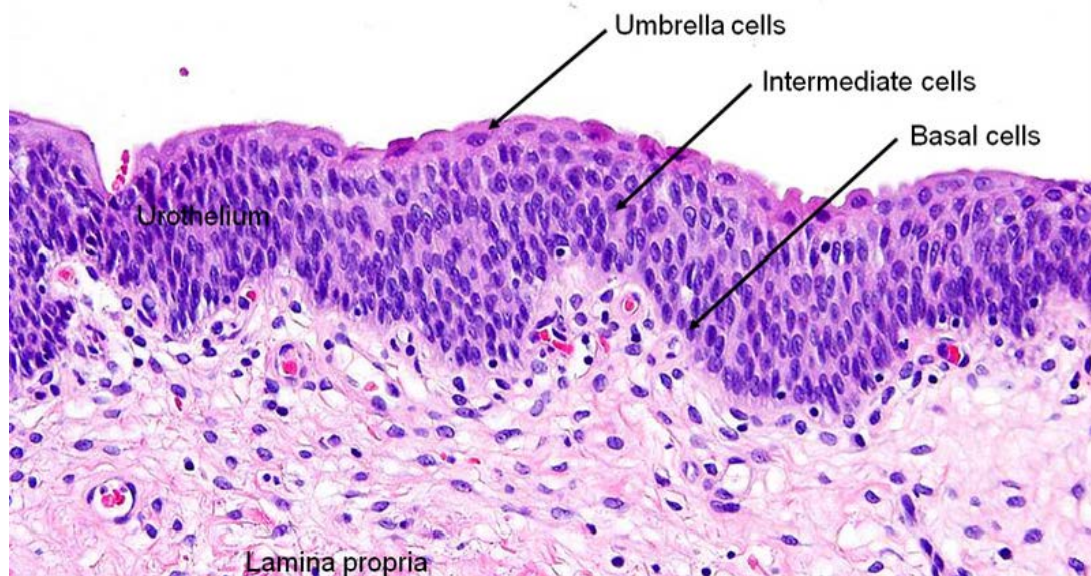


Figure 1.2 – Transitional epithelium that lines urinary bladder [12].

1.3 Urinary catheterisation

The word catheter is derived from a Greek word *kathiénai*, which means “to thrust into or to send down” [8] and its use can be dated back to 1500 BC when bronze tubes or straws were used to treat urinary retention [13]. Up until the invention of modern day catheters the sole purpose of catheter was to treat urinary retention in the male [8]. However, in the later years, hollow tubes were not only used to drain bladder but also to insert pharmacological products into urinogenital tract. The development or improvement in the design of catheter was not seen until 1500s when the first wax-impregnated cloth catheter was made to lessen the severity of damage caused by inserting hard metallic tubes [14]. The modern-day catheters, termed as Foley catheters, were developed in 1929 and are described in section 1.3.1.

Nowadays catheterisation is an invasive procedure, where a rubber tube is inserted into urinary bladder via urethra for a continuous flow of urine into a collecting system. It is frequently used in hospitals and nursing homes for management of several conditions. Many patients who are admitted in hospital require urinary catheterisation at some stage during their stay. There are various reasons for catheterisation ranging from collecting a sterile urine sample to treating long term underlying conditions such as urinary incontinence due to bladder weakness or nerve damage. Catheters are also used in patients undergoing surgery or suffering from conditions such as multiple sclerosis, enlarged prostate and spinal cord injury [15]. Urinary catheters are also indicated for measuring bladder residual volume, obtaining urine samples and delivering drugs [16, 17].

1.3.1 Types of catheters

Depending upon the need there are variety of catheters available, however, the two main types of catheters are intermittent and indwelling catheters. **Intermittent catheters** are single use catheters that are used either to collect sterile urine samples or where patients are unable to empty their bladder properly therefore a catheter is inserted to drain urine. In such cases, once the bladder

is full, a catheter (typically a long plastic or rubber tube) is inserted aseptically into the urethra until the urine starts to flow [8]. Once the flow has stopped the catheter is removed and disposed of. The procedure needs to be repeated several times in a day. It is also used to manage patients with spinal cord injury or neuromuscular degeneration.

Indwelling catheter is the most common type of catheter that is inserted in the same way as the intermittent catheter however left in the bladder for a long period. Indwelling catheters can be inserted via urethra into the bladder or by making a small incision in abdominal wall. Depending upon the underlying medical condition and the need, indwelling catheters are inserted for short or long term. When a catheter is inserted for few days i.e. less than 14 days, it is classed as short-term catheterisation. On the other hand, long term catheterisation has been categorised differently by different studies, however majority of them classified catheterisation as long term if catheter is in body for more than 30 days and up to 90 days [4, 8, 16, 18].

The most widely used catheter these days is a **Foley catheter** (Figure 1.3), designed by American urologist, Frederick E.B. Foley [8]. Originally this catheter was designed to drain blood followed by prostatectomy, however soon it was used for management of urinary retention and incontinence. A Foley catheter is a thin, hollow tube made of soft, flexible material that is passed through the urethra into the bladder. The size of the catheter is measured in Charrierers or French Grade (1Ch =1/3mm diameter) and adult catheters are available in different sizes and lengths depending upon factors such as age, gender, purpose and the overall health of urinary tract [19, 20]. A typical Foley catheter size is 16 Ch (between 10 and 24 Ch) and 220 - 380 mm in length [21]. It uses a retention device at proximal end such as a water filled balloon to keep the catheter in place. It has two or three different channels; one for inflating balloon and the other for draining urine or delivering medications. Once *in situ* the balloon is inflated/deflated with sterile water using a syringe. Historically buckets were used for collecting urine, however a closed drainage system such as a bag is attached to the urine outlet and is either hung beside the patient's bed or strapped to the patient's leg (Figure 1.4). Urine can either drain freely by gravity into the drainage

bag or a catheter valve can be fitted into the end of the catheter which may be switched on or off to allow for the urine to drain.

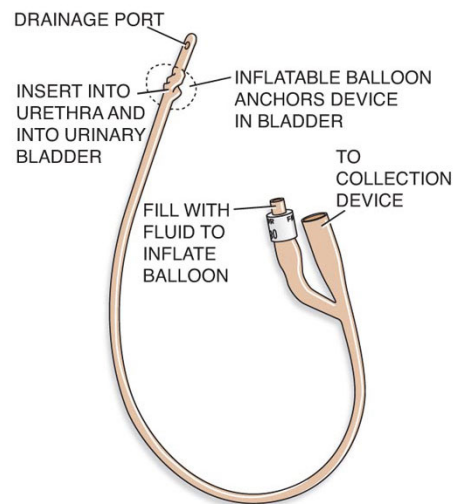


Figure 1.3: A Foley catheter [22].



Figure 1.4: A Foley catheter inserted *in situ* – a closed drainage system [23].

The first Foley catheter was made from latex, however nowadays they are made from many different materials to increase their biocompatibility and functionality. Like all devices biocompatibility of a catheter is highly dependent on the surface characteristics and its chemical nature. Whilst there is no ideal material for catheters some materials are more biocompatible than others. Latex is inexpensive, has good elastic properties but tends to be more prone to bacterial adherence and have more allergic potential than some other materials [24, 25]. The cytotoxic effects of latex were reported due to eluted components from rubber [26]. PVC has been used in the past, however it elicits an acute inflammatory response *in vivo* due to which these catheters are outdated. Silicone on the other hand, is soft, non - irritating and clinically stable making it ideal for long - term use in the urinary tract. It is more expensive than latex catheters but is inert, putting patients at less risk of allergic reaction. Their surface properties make catheter insertion easy and lower rates of bacterial adhesion and encrustation have been reported compared to latex catheters. This has been attributed to the smoother surfaces of these catheters exhibiting less niches for microbial aggregation than latex catheters [27, 28].

1.4 Complications associated with catheterisation

It was not until 1988 when an American physician Calvin M. Kunin published in the New England Journal of Medicine, that the use of Foley catheters was considered safe [29]. There are several complications associated with catheterisation that limit the use and effectiveness of catheters in healthcare. Discomfort, trauma, bladder spasm, overactive bladder, anaphylaxis due to latex allergy, cytotoxicity, hematuria, intravesical knotting, urethral fistulae, urosepsis, symptomatic infection and encrustation are among the most common complications due to catheterisation [30, 31]. The focus of the given study is prevention and treatment of infection due to urinary catheters.

1.4.1 Encrustation

One of the major complications of urinary catheterisation is encrustation that causes blockage or obstruction of catheters causing retention of urine in the bladder. This subsequently causes reflux to upper urinary tract or leakage of urine. It occurs because of crystallisation of the ionic components in the urine on the surface of an indwelling catheter initiated by certain bacteria [32]. About 40 - 50% of the patients undergoing long term catheterisation experience blockage of the catheter [3, 4, 8, 33], which can occur outside or inside lumen of the catheter. The main cause of catheter encrustation is colonisation of urinary tract by urease producing bacteria and their activity is pH dependent. Urease is an enzyme, that catalyses the hydrolysis of urea in the urine to ammonia and carbon dioxide [34]. The production of ammonia increases the pH of the surrounding environment that initiates precipitation of magnesium and calcium salts within the urine to struvite and hydroxyapatite respectively [35]. Meanwhile bacterial biofilms are also formed and the crystals are deposited forming crystalline biofilms which continues until the eye hole of the catheter and lumen is blocked preventing the flow of urine through catheter to collecting bag.

There are several species associated with CA-UTIs that are urease positive such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus* species and some strains of *Staphylococcus aureus* [18, 36, 37], however the most commonly isolated microorganism from crystalline biofilms is *Proteus mirabilis* [35]. The enzyme produced by *Proteus mirabilis* is highly potent and has ability to cause crystallization several times faster than other bacteria. According to an analysis conducted by Sticker *et al.* other bacterial species (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Morganella morganii*) are less capable of producing alkaline urine and hence failed to form crystallization on catheters compared to *Proteus mirabilis* and *Proteus vulgaris*. The former is shown to form crystalline biofilms on all types of indwelling catheters including silver and hydrogel coated catheters [33]. Even if the blocked catheters are changed, in some cases recurrent blockage occurs due to presence of *Proteus mirabilis*. A study

conducted by Sabbuba *et al.* [38] on bacterial strains isolated from urine samples, demonstrated recurrent bacterial infection and encrustation by *Proteus mirabilis*. The researchers identified a patient with persistent *Proteus mirabilis*, even though the patient had a course of antibiotics, eight catheters changed and 20 days without catheter, the infection continued for four months.



Figure 1.5: A catheter that had been indwelling for 6 months **(a)**. A cross section of silicone catheter removed after 8 weeks **(b)**. A longitudinal section of a silver/hydrogel-coated latex catheter that had blocked after 11 days **(c)** [35].

1.4.2 Infections

One of the most common complications of urinary catheterisation is catheter associated urinary tract infections. The infection is a microbial colonization of the urinary tract by pathogenic microorganisms causing signs and symptoms of inflammation when a catheter is present. The infection is said to be present if the urinary bacterial or fungal count is more than 10^3 CFU/ml

[39]. According to Foxman [40], the infections are classified according to the site of infection as the bladder (cystitis), kidney (pyelonephritis) and urine (bacteriuria).

1.4.2.1 Epidemiology

Urinary tract infections (UTI's) have been reported as the most common health-care associated infections where 80% of these infections are due to insertion of a urinary catheter. Due to frequent and sometimes unnecessary use of catheters, CA-UTIs, equate for 8-35% of healthcare-related infections [27, 39], and are the second most common cause of septicaemia which is associated with substantially increased institutional death rates [41-43]. Studies have shown that about 15-25% of the patients admitted in hospital require urinary catheterisation [27] which increases their risk of developing urinary tract infection by 5% per day [18, 39]. In about 30 - 40% of the patients suffering from CA-UTI microorganisms may enter blood and progress to septicaemia, which accounts for 8% of all nosocomial infections [27]. 1 in 3 patients who acquire bacteraemia in blood will eventually die of the infection [44].

CA-UTIs are frequently associated with significant morbidity. A detailed review of the literature on morbidity and mortality associated with complications arising from catheter insertion was undertaken by Delimore and co-workers [21]. The study shows 100% morbidity rate in patients with CA-UTIs, when inserted for more than 30 days. The study also established symptomatic bacterial infection as the most fatal complication directly occurring due to catheter insertion. The high mortality rate, approximately 21%, was reported in older adults who required an indwelling urinary catheter. Another recent study conducted on patients suffering from nosocomial bacteraemia showed a high mortality associated with CA-UTIs. The authors compared central venous catheters (CVC) associated bacteraemia with catheter associated bacteraemia and found significant death rate within seven days of inserting a urinary catheter [45]. Similarly, a prospective study conducted by Platt *et al.* [46] on urinary catheterisation and associated mortality showed a 3-fold increase in the death rate, however many other studies argue on the association

of death with urinary catheterisation. Thus, the effect of catheter associated UTIs on mortality is unclear as patients requiring catheters are generally suffering from severe illness, therefore the death could be due to underlying conditions and not the CA-UTI.

1.4.2.2 Financial costs

Even though the problem of CA-UTIs is well recognised in health care settings, the full extent of the problem is not established. Nevertheless, the financial implications are enormous as the high complication rate arising from catheterisation requires significant time and cost. The cost is substantial due to extended length of stay at hospitals, nursing visits, antibiotics. In the UK, in 2000 it was estimated that the management of catheter associated infections cost £120 to £2600 per patient for each quarter of a year [47]. The findings of Plowman in 1999, reported that an average increased stay of 5-6 days resulted in £1327 spent extra on each case [48]. More than a million cases are reported each year accounting for annual economic burden of approximately £125 million [3, 39]. The cost also includes extra expenses associated with diagnostic tests and treatment regimes.

1.4.2.3 Risk factors

Several factors play a key role in development of CA-UTI such as previous history of catheterisation, reason and the site of insertion [39]. Duration of catheterisation is a major risk factor associated with CA-UTI. The longer a patient requires catheterisation, the greater the chance of developing the associated infection [40]. According to Garibaldi *et al.*, [49] the risk of developing bacteraemia increases 5% for each day the catheter is in body and by the tenth day, 50% of the patients develop CA-UTI and 100% patients contract infection by the day 30.

Among the other factors is increasing age and gender. The relative rate of CA-UTI is reported to be much higher in females than in males [39], and this is due to the relative ease with which

bacteria can travel to the urethra from the perineum. Estrogens promote growth of vaginal lactobacilli which prevent growth of enterococci [50], therefore the risk of CA-UTIs increases in menopausal women due to low levels of estrogens. Patients with other site infection, chronic health conditions such as diabetes, malnutrition and renal inefficiency are also at a risk of developing CA-UTI [39]. In addition, catheterisation in unsterile environment, use of unsuitable catheters and open drainage systems increase the incidence of CA-UTIs.

1.4.2.4 Causative agents

Catheter associated urinary tract infections are caused by a diverse range of microorganisms, both bacterial as well as fungal species. The spectrum of microorganisms isolated from the urine samples varies not only within patients but also some microbes are unique to each facility. Majority of the infections are caused either by endogenous skin microorganisms or faecal contaminants residing in periurethral area. The most common of the bacterial species is *Escherichia coli* [4, 51] whereas *Candida albicans* is also frequently isolated [52-54]. Among the other species that are commonly isolated from the samples are *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* spp (mostly *E. faecalis* and *E. faecium*), and *Staphylococcus* spp. (mostly *S. aureus* and Coagulase Negative *Staphylococci*). *Proteus* and *Pseudomonas* species are most commonly associated with biofilm formation on the catheter surface [4, 55]. These microorganisms especially *Proteus mirabilis* are not isolated following initial catheter colonisation and therefore not common in patients undergoing short term catheterisation. According to Nicolle, 50% of the infections in short term catheterised patients are due to *Escherichia coli* whereas long term catheter infections are mainly polymicrobial and up to 40% of infections are due to *Proteus* species [56].

Melzer *et al.*, conducted an analysis from October 2007 to September 2008 on patients with hospital acquired bacteraemia and established CA-UTIs as the second most common HAI [45].

Among the patients with CA-UTI, 92.7% of the isolates were Gram negative and 7.2% were Gram positive. The proportion of each isolate is given in the chart below (Figure 1.6).

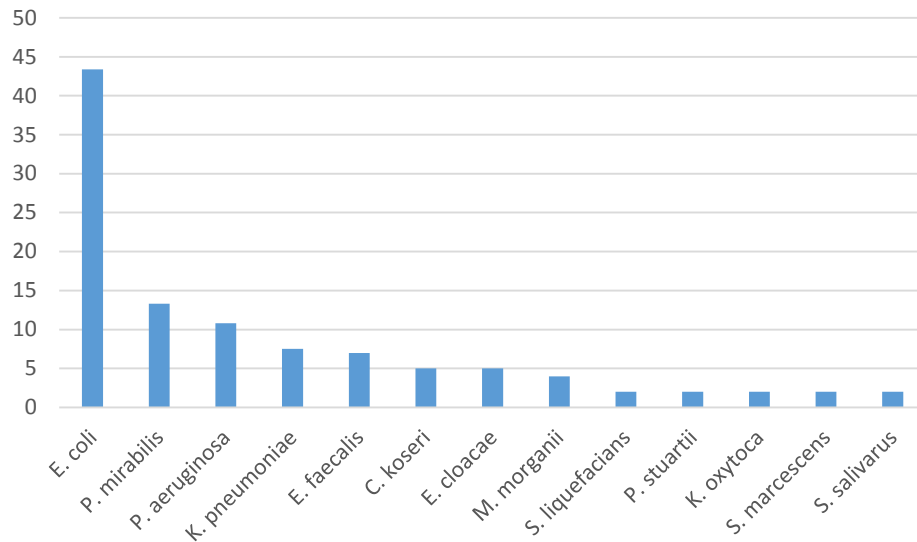


Figure 1.6: Proportion of bacteriaemic isolates causing catheter-associated urinary tract infections [45].

1.4.2.5 Routes of entry

The urinary tract, like most internal organs is sterile (apart from the distal tip of the urethra), however under certain conditions microorganisms gain access to the bladder and kidneys. Insertion of a urinary catheter is the major route by which microorganisms may ascend the urinary tract and therefore cause infection. When a catheter is inserted microorganisms can enter the urinary tract by the contaminated hands of the person carrying out the procedure [39]. Most pathogens that cause CA-UTIs are faecal contaminants or skin residents of the patient's own microflora that colonise the perianal area. Once a catheter is inserted, microorganisms travel along the surface of the catheter extraluminally or intraluminally into the bladder urethral meatus [57]. The preferred mechanism is extraluminal. In 66% of the cases microorganisms ascend via the urethral-catheter interface, whereas 34% of the cases are due to intraluminal migration

associated with manipulation of collecting systems or due to contamination of the drainage bag or disruption of the catheter tubing junction [3, 57]. Microorganisms gain access in the urinary tract using motility organs such as flagella and pilli, fimbriae or other adhesins, on the other hand microorganisms without such appendages use Brownian motion [58].

1.4.2.6 Pathogenesis/Biofilm formation

Normal human urinary tract has innate protection against a variety of pathogens that enter the system, however once a foreign body such as a catheter is inserted, it increases the risk of developing UTI's. Catheter surface provides an attachment site and favourable conditions for microorganism to colonise and infect the uroepithelial mucosa thus leading to biofilm formation. *"A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material"* [59]. A detailed account of the pathogenesis and formation of biofilm is given below:

Once a catheter is *in situ*, it is exposed urine. The urine contains a variety of components such as proteins, glycoproteins, electrolytes and other organic molecules, which adsorb onto the surface of catheter forming a **conditioning film**. According to Keane *et al.*, [60] the conditioning film does not cover the catheter surface completely instead it forms a mesh like covering. The deposition of conditioning film is crucial as it not only masks the surface composition and properties of the catheter material but also contains host cell binding receptors which are primary attachment sites for microbial colonisation. In addition, the presence of urinary catheter impairs the normal host defences, such as physically damaging the protective uroepithelial mucosa leading to exposure of more binding sites. Likewise, normal mechanical defences of the bladder (urine flow) are also disrupted which results in an over distension and incomplete voiding that leaves residual urine in bladder hence enhancing microbial growth [61]. Once the conditioning film is formed, microbial colonisation begins by means of hydrophobic and electrostatic interactions and by means of **adhesins**. Microorganisms have special cell surface components

that facilitate anchorage of the cells to surfaces. Gram negative bacteria such as *E. coli* have a diverse range of adhesins such as flagella, pili and fimbriae, whereas *P. mirabilis* produce MR/K type fimbriae and haemagglutinins. MSCRAMM's (microbial surface components recognizing adhesive matrix molecules) are the adhesion proteins that enable attachment of *S. aureus* on catheter surfaces. Even though the presence of a catheter surface is an advantage for microorganisms, certain other factors such as the host immune response and surrounding flow rate of the urine also play a key role in successful microbial colonisation. Initially the attachment is reversible, however irreversible attachment occurs when bacteria begin to change phenotypically by upregulating expression of certain genes [3] . Upregulation of these results in production of exopolysaccharides which protect not only bacteria from immune components but also antibiotics and shear force. These attached bacteria reproduce and form colonies that ultimately mature into biofilms (Figure 1.7 and 1.8). Channels are formed to permit exchange of nutrients and waste products [59]. The biofilms formed shed daughter cells that spread into other parts of catheter and form biofilms there.



Figure 1.7: illustration of biofilm formation. Planktonic cells adhere onto the surface of catheter containing host cell proteins (within conditioning film) and secrete matrix components resulting in biofilm formation [62].



Figure 1.8: Scanning electron micrograph of *Staphylococcus aureus* on the luminal surface of an indwelling catheter. The round cocci shaped bacterial cells are interwoven in a complex matrix of exopolysaccharides known as biofilm [63].

1.4.2.7 Symptoms

Although the presence of bacteraemia is believed to induce an inflammatory response, most cases of CA-UTIs are asymptomatic i.e. 90% of patients suffering from CA-UTI did not show any symptoms [39]. These patients do not require any treatment unless the infection show symptoms or the patient is immunocompromised. However symptomatic cases range from mild fever and cystitis to severe cases of acute nephritis. The most common signs are abnormal urine colour, foul odour, dysuria (pain during urination) and urgency. Among other symptoms include pain in lower abdominal area, chills and confusion. If left untreated, CA-UTIs can lead to abscess formation, renal obstruction, scarring and eventually will lead to septicaemia and possibly death [3].

1.4.2.8 Treatment

Asymptomatic infections are considered as clinically insignificant therefore treatment is unlikely to confer benefit. However, many patients are prescribed antibiotics unnecessarily which not only adds to antibiotics resistance but also inappropriate costs to the NHS. Symptomatic infections with one type of organism are treated with:

- Trimethoprim – effective against most uro-pathogens except for *Pseudomonas* and *Enterococcus* species
- Fluoroquinolones - effective against many Gram negative such as *Pseudomonas* and *Proteus* species
- Nitrofurantoin - effective against most uro-pathogens except *Pseudomonas* and *Proteus* species

Cephalosporin is used for patients with polymicrobial infection whereas a combination of drugs is prescribed for complicated infections. Biofilms are unlikely to clear with antibiotics, even if the catheter is changed there is always a risk of reoccurrence of infection and there remains a need to develop preventive strategy to reduce the occurrence of catheter associated infections.

1.4.2.9 Prevention

There is very little that can be done to prevent catheter associated urinary tract infections. The first and the foremost precautions is hand hygiene and aseptic technique while inserting a catheter in the bladder. Health care staff and clinicians should be trained to prevent transmission of infection by adopting the hygiene guidelines such as use of antiseptic soaps and alcohol hand rubs. Whilst the benefit of handwashing has been proven effective in many studies, lack of consistent hand washing practice, as demonstrated by Collins [1] where on average only 40% of healthcare staff adhered to recommended hand hygiene procedure, could aggravate the problem. In addition to hand hygiene a closed drainage system and minimising the duration of catheter

usage has been shown to reduce the infection rate. It is recommended to change the catheter as per clinical need or according to the manufacturer's guidelines, which is usually at 10 - 12 week intervals [34]. Systemic antibiotics are advised in certain cases however have shown little or no benefit as it only delays the infection. The use of antibiotics however promotes antibiotic resistance and are not recommended even for the treatment of bacteriuria in the asymptomatic catheterised patient [64].

Selective and limited use of catheters is critical in preventing CA-UTIs nevertheless under certain circumstances the use of catheters is inevitable. As adhesion to the catheter is crucial to the establishment of microorganisms and biofilms [3] therefore one of the prevention strategies is use of a catheter that prevents attachment of microorganisms onto the catheter surface. To prevent bacterial attachment on the surface of catheters an array of biomaterials such as C-Flex™, latex, nylon, Percuflex™, polyethylene, polyvinyl chloride, polyurethane, silicone, Tecoflex® have been developed in the past, however most of these materials were directed to reduce friction. While catheters are coated with a variety of materials such as hydrogel, silicone and hydrophilic coatings to reduce frictions and inhibit microbial growth, the inability of these materials to resist bacterial adhesion has led to the development of catheters with antimicrobial agents [21].

1.5 Antimicrobial catheters

Antimicrobial catheters were developed in the past to prevent bacterial colonisation on the surface of catheters. For instance, chlorhexidine containing catheters have shown *in vitro* activity against *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus epidermidis* [65, 66]. Similarly, hydrogel coated catheters were loaded with bacteriophages to prevent bacterial growth. This was achieved by exposing the catheter segments to a phage culture such as lytic *Staph. epidermidis* bacteriophage 456 for a defined period. According to Carson *et al.*, [67] hydrogel coated catheters containing bacteriophages reduced 99.9% of CA-UTIs caused by *Escherichia coli*. Similarly,

Staphylococcus epidermidis phage had been shown to reduce 4 logs over 24 hours [68]. While some successful cases have been reported, the ability of bacteria and viruses to mutate in short time, high phage specificity and immune response of the host are the factors that caused concerns over their usage. Among the other technologies that have been developed in the past is light activating antimicrobial agents (LAAA) [69]. These agents can produce reactive oxygen species (ROS) which bring about cell death by targeting several cell components. Methylene blue incorporated in polymers has also shown significant reduction in *E. coli* and MRSA within five minutes of irradiation [69].

Broad antimicrobial spectrum antimicrobials have been incorporated in urinary catheters to prevent catheter related infections and biofilm formation. The antimicrobial catheters that were developed and used in clinical practice included antiseptic coated catheters (silver oxide or silver alloy coated) and antibiotic-impregnated catheters containing nitrofurazone or a combination of the broad-spectrum antibiotics, minocycline and rifampin [70]. However, according to various studies these catheters, which initially showed promising results, did not significantly reduce the risk of CA-UTIs in large, well-controlled trials and are still the subject of considerable controversy [3].

A detailed account of antimicrobial catheters is given below:

1.5.1 Antibiotic containing catheters

Antibiotic-impregnated catheters were developed to treat a broad range of microorganisms; however, these catheters did not show promising results due to development of resistant bacterial strains. Darouiche *et al.*, [71] conducted a study on short term catheterisation of post-operative patients. An antibiotic impregnated catheter with minocycline and rifampicin was used. The catheter was shown to successfully reduce the rate of growth of Gram positive bacteria over a two-week period however failed to inhibit colonisation of Gram negative bacteria such as

Escherichia coli and *Proteus mirabilis*, which are majorly associated with infection and encrustation. Whilst the catheter showed some positive results, it was believed to linked with possible antibiotic resistance in Gram positive bacteria [71].

1.5.2 Nitrofurazone

Nitrofurazone (NF) is an antimicrobial agent that belongs to nitrofurane group of antimicrobials and has a wide range of activity against microorganisms associated with CAUTIs. A nitrofurazone impregnated catheter called ReleaseNF was developed by Rochester Medical Corporation, USA. It is a 100% silicone, two way Foley catheter which works by eluting NF, both intraluminally and extraluminally, into the urinary tract [72]. The NF is impregnated in both sides of the catheter. The released NF acts as a bactericidal agent and kills planktonic bacteria before they develop into biofilm [72]. The efficacy of this type of catheter in reducing CAUTIs has been evaluated by several laboratory studies and clinical trials, of which a few are discussed here.



Figure 1.9: ReleaseNF Foley catheter coated with nitrofurazone [72].

An *in vitro* study to evaluate the effectiveness of NF containing catheter compared to a standard silicone catheter was conducted by Devak and co-workers in 2011 [73]. In the given study adherence of two pathogens; *Escherichia coli* and *Enterococcus faecalis*, on different catheter surfaces in broth was evaluated. The study showed significant decrease in *Escherichia coli* colonisation on the NF catheter compared to the silicone catheter as there were no bacteria found on the catheter material and surrounding environment. Even though statistically significant differences in the bacterial load were seen between NF catheter and standard silicone catheter, the inhibitory effect decreased with incubation time; 100%, 97%, 91%, 62%, 31% on day 0, 3, 5, 7 and 10 respectively. On the other hand, NF catheter lost inhibitory effect against *Enterococcus faecalis* after 3 days. Among the other prominent study is one published by Johnson *et al.*, [42] which demonstrated the effectiveness of NF catheter against 86 susceptible and multidrug-resistant (MDR) clinical isolates (*Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Staphylococcus aureus*, coagulase-negative staphylococci, and non-vancomycin resistant *Enterococcus faecium* [74, 75].

A prospective double blind trial conducted by Maki *et al.*, [39] showed a significant threefold reduction in the rate of CAUTI with a nitrofurazone impregnated catheter compared to standard silicone catheters for the first five days. In another study by Leclair [76] nitrofurazone catheters introduced in burns intensive care units reduced the catheter associated infections from 24.4 per 1000 catheter to 7.5 per 1000 catheters. Similarly, Stensaballe *et al.*, [77] conducted a double randomized controlled trial and showed significantly less infection rate in nitrofurazone group compared to control group. Whilst many clinical trials and *in vitro* analysis suggest effectiveness of nitrofurazone containing catheters, the data however show the use of catheter is only beneficial in the first five days of catheterisation [78].

1.5.3 Silver containing catheters

Silver has been used for many decades for its antimicrobial properties, with a variety of applications ranging from treatment of conditions such as burns, abscesses and other types of infections and as part of wound dressings [79]. It has a broad antimicrobial spectrum and does not promote bacterial resistance. Silver exerts its activity by releasing silver ions in surrounding environment which have a bacteriostatic action at low concentrations (0.1µg/ml) and are bactericidal at high concentrations (1.6µg/ml) [80]. Silver has been used in a range of medical devices and in many ways to reduce CAUTIs. An *in vitro* analysis was undertaken by Veldhoen *et al.* [81], where silver was shown to inhibit colonisation of the urinary tract by *Proteus mirabilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. The inhibition was only seen for the first 10 days of catheter insertion compared to experimental models with no silver where colonisation was seen approximately after five days.

Silver containing catheters are available as silver alloy coatings or as silver oxide. Many studies; *in vitro* and clinical trials have been performed to evaluate the efficacy of silver containing catheters compared to standard catheters. Two commercially available silver containing catheter available in the UK are the Bardex IC and the Dover Silver catheter. The Bardex IC catheter is manufactured by Bard and contains silver with gold and palladium (Figure 1.10) whereas the Dover silver catheter is coated with hydrogel containing silver [82]. The **Bardex IC** catheter claims to have a precise balance of elements i.e. silver, gold and palladium that are released at a constant rate to prevent the microbial adherence and biofilm formation [83]. According to the manufacturers the catheter reduces the rate of CA-UTIs up to 47%.



Figure 1.10: Bardex IC [83].

Johnson *et al.* [42] conducted a clinical investigation on 482 acutely hospitalized patients to compare efficacy of **silver oxide coated** catheters with uncoated catheters. The study showed a reduce rate of CA-UTIs in female patients (19% for control verses 0 for silver oxide coated catheters) compared with a control silicone catheter. The efficacy of **silver–alloy** catheters has also been evaluated but showed conflicting results. Different results have been reported by various authors, for instance Rupp *et al.* [84] reported that use of silver alloy coated catheter decreased the rate of infection compared to the standard latex based catheter. They reported that the incidence of CA-UTIs decreased from 6.13/1000 to 2.6/1000 over a 2-year period. Similarly, Schumm and Lam [85] reported a significant decrease in the risk of asymptomatic bacteria when they used a silver alloy catheter, for up to two weeks. However, a randomized study conducted by Riley *et al.*, [86] on 1,309 catheterised patients showed that 5% silver oxide coated catheter did not have antimicrobial effect compared to a standard catheter. Surprisingly silver oxide coated

catheters showed an inexplicable increased incident of bacteriuria in male patients with a high occurrence of *Staphylococcus*.

Niel-Weise [87] reviewed seven clinical studies that compared silver oxide coated catheters with standard catheters, however did not find sufficient evidence to recommend the use of these catheter. Similarly, in a study conducted by Srinivasan *et al* [88] on silver-silicone hydrogel showed no benefit of silver in preventing bacteriuria. While some studies strongly suggest benefits of silver coated catheters, many others [89-91] question the quality of the trials. They criticised that the fundamental criterion for infection is inconsistent, lacking the distinction between UTI and asymptomatic bacteriuria, as symptomatic infection is of clinical interest not asymptomatic UTI.

1.5.4 Limitations

Several studies and reviews have been undertaken on efficacy of antimicrobial catheters, however the effectiveness of these catheters is still not proven. The lack of activity of antimicrobial catheters could be due to several reasons.

1. Deposition of conditioning film on the catheter surface protect microorganisms from getting in direct contact with antimicrobial surface or components in the catheter. This was shown in studies where bacterial colonisation was seen shortly after deposition of a conditioning film [92, 93].
2. The release rate of the antimicrobial components from the catheters also plays a role. The antimicrobial efficacy of the coatings is highly dependent on the mechanism by which active components are incorporated in to the devices which subsequently affect the uniform dissolution of the ions into the surrounding aqueous environment. Thus, the lack of activity could be either antimicrobial components (e.g. Ag⁺) are not released from the coatings or

the coating itself is washed off relatively quickly hence provide the chance for bacterial colonisation [94, 95].

3. Furthermore, certain materials such as the hydrophobic silver-oxide coatings, discourage dissolution which explains the lack of antimicrobial activity of silver ions [96].
4. The efficacy of the released antimicrobial ions can be affected by the presence of certain organic and inorganic components in the local environment. For instance, it has been shown that the effectiveness of silver is reduced in the presence serum, chloride ions and albumin [95].

Studies have been undertaken that provide sufficient evidence that antimicrobial catheters can delay or prevent colonisation in short term catheterised patients, however there is no proven benefit of using these catheters in long term catheterised patients. *Proteus mirabilis* has been shown to migrate across and form biofilms even on silver containing catheters when inserted for long term [3, 33, 97, 98]. To conclude, technological innovations have been introduced and evaluated in past two decades, however unsuccessful trials of antiseptic coated catheters and development of resistance in antibiotic impregnated catheters calls for development of new catheter coatings. Therefore to achieve clinically significant results there is still a need for development of an antimicrobial catheter, that will prevent bacterial adhesion for an extended period (>30 days) with a sustained release of antimicrobials.

1.6 Bioactive glass - A delivery system

Glasses could potentially be used to provide a controlled release of antimicrobial ions. Bioactive material, as the name indicates, is one that *elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material* [99-101]. Structurally a glass is similar to a liquid but since they have high viscosity they are classified

as solid materials. Glass is defined as *a solid that possesses a non-crystalline structure and exhibits a glass transition temperature when heated towards the liquid state* [99]. Bioactive glasses therefore are a class of biomaterials that elicit a biological response at the interface of the material which stimulates cell proliferation, differentiation and forms a bond between living tissue and the material [101-103]. The first bioactive glass, later termed as 45S5, was invented by Professor Larry Hench in 1969 as an alternative to inert implants and since then many new materials with various glass compositions have been investigated. A brief account of glass synthesis, structure and applications is given below.

1.6.1 Synthesis

Bioactive glasses can be classified into two categories based on the method used to synthesize them; melt-quench and sol-gel. In the melt quench method the raw materials are heated up to high temperatures usually between 1200-1500°C, in a platinum crucible for a period [104]. This creates a melt which if cooled at a sufficiently high rate prevents crystal growth resulting in glass formation. To suppress crystallisation, the melt is poured in a pre-heated graphite mould or water and cooled slowly to room temperature to remove any residual stress. [105]. On the other hand, sol gel glasses are prepared by a chemical based process in which glasses are prepared by a polycondensation reaction from organic precursors (alkoxides) [105]. Sol-gel glasses, unlike conventional melt-quench glasses, require lower temperatures.

The manufacturing process of the melt-quench and sol-gel glasses results in some differences in the glass structures. Sol-gel glasses have an inherent nanoporosity whereas melt-derived glasses are dense in nature [106, 107]. Sol-gel glasses tend to have higher dissolution rates due to higher surface area and hence higher bioactivity compared to the melt-quench glasses. The first bioactive glass, 45S5 was made using melt-quench method and since then most of the glasses to date are made using this method. This is because the technique is simple, low cost and requires less time to complete in contrast to sol-gel glasses. Additionally, the sol-gel application of Na₂O-

containing bioactive glasses is limited due the high hydrolytic reactivity of sodium alkoxide in water [108]. A schematic representation of glass synthesis via melt-quench and sol-gel method is given in Figure 1.11.

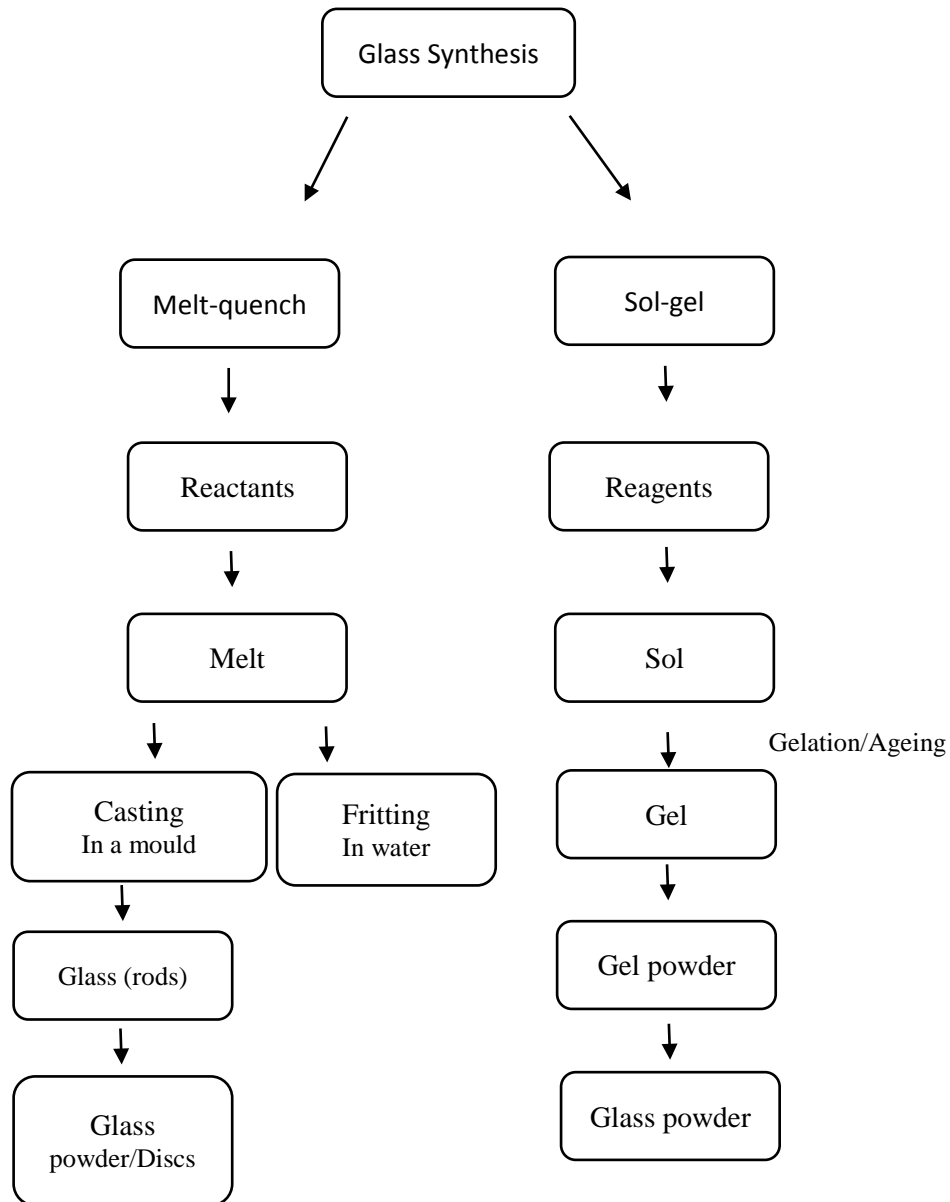


Figure 1.11: Schematic representation of glass synthesis via melt-quench and sol-gel method.

1.6.2 Structure

Glass is an amorphous solid that lacks a long range order or symmetry of atomic arrangement and exhibits time-dependent glass transformation behaviour [105]. Glass transition behaviour can be described based on a volume-temperature diagram (Figure 1.12). When a liquid is cooled to any temperature below the melting temperature, T_m , it will result in the conversion of the material to the crystalline state with long range periodic atomic arrangement [109]. However, if the cooling is quick enough, crystallization is inhibited and a glass will be formed. The glass transformation or glass transition occurs over a range of temperatures T_g and cannot be characterized by any single temperature [109].

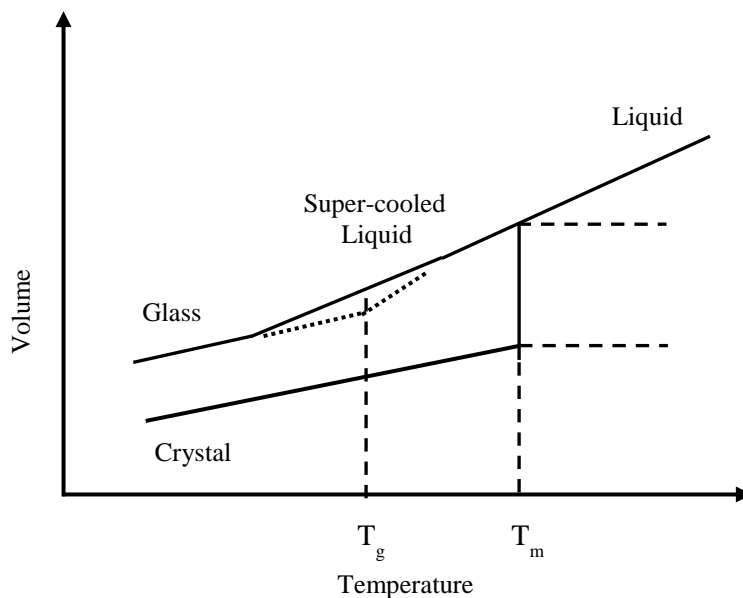


Figure 1.12: Schematic representation of glass or crystal formation from liquid melt. T_g (glass transition temperature), marks the transition of the super cooled liquid to a glassy state where as T_m represents the melting temperature. Adapted from [110].

A glass is a network of atoms bonded to oxygen via covalent bonds. Three different components are described within in glass structure; network former, network modifier and intermediate oxides. It is possible to make a glass with network former such as silica (SiO_2), phosphorus pentoxide (P_2O_5), and boron trioxide (B_2O_3) without the need for additional components. For instance, silicate glass consists of silica tetrahedron (SiO_2) as the building units, that are connected to each other by oxygen atoms ($-\text{Si}-\text{O}-\text{Si}-$), commonly termed as bridging oxygen atoms (BO). Thus, in a vitreous silica each tetrahedron is linked to another by each of its four corners corresponding to four BOs per tetrahedron. In other words, vitreous silica has a network connectivity (NC), defined as the average number of BO atoms bound to a network-forming cation, of four, that decreases when network modifiers are added.

The addition of network modifiers, such as sodium and calcium oxides cause cleavage of covalent $-\text{Si}-\text{O}-\text{Si}-$ bonds, which results in formation of non-bridging oxygen atoms (NBOs). Thus, the resulting glass structure not only contains covalent bonds but also ionic cross-links between NBOs (Figure 1.13). The third class of glass components is the intermediate oxides. These oxides can switch their role, depending upon the composition, as network former or modifier. Examples include magnesium, iron, aluminium, zinc and titanium etc.

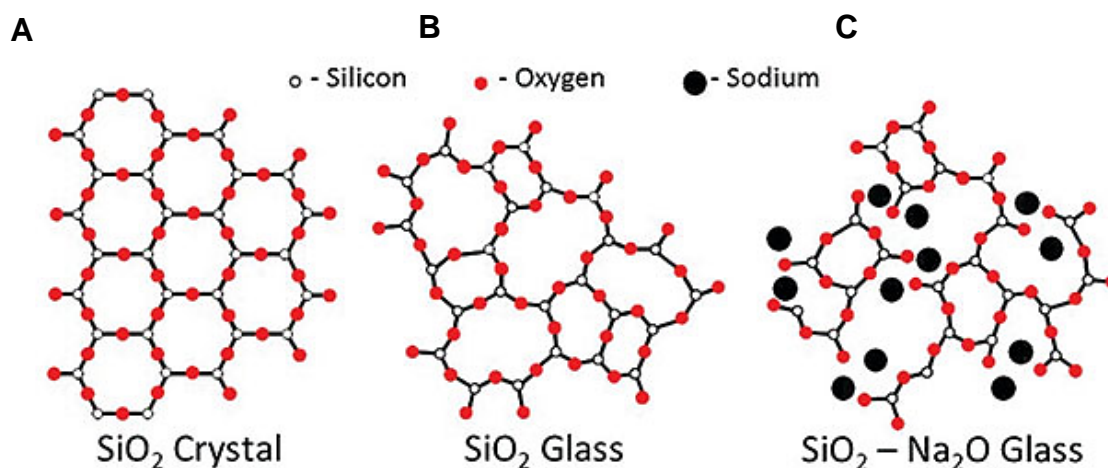


Figure 1.13: (A) Two-dimensional structure of crystalline silica and (B) vitreous silica. The network periodicity and symmetry of crystal silica differs from the disorder seen in the glass network. (C) Two-dimensional glass structure with network modifiers [111].

1.6.3 Bioactivity

The bioactivity of glasses, as described earlier, is their ability to elicit a specific biological response in the human body. The bioactivity of glasses can be estimated based on their composition i.e. the content of glass formers and modifiers. For instance, in silicate glasses if the silica content is greater than 60 mol % a highly-connected network is formed which results in low dissolution and hence low bioactivity. However, addition of calcium and sodium ions opens the glass network allowing better penetration of water molecules thus facilitating glass dissolution and hence bioactivity. Bioactivity map of soda – lime – phosphosilicate glass is shown in Figure 1.14, glasses in region A bond to bone and thus are bioactive whereas glasses in region B are bio-inert and would result in scar tissue encapsulation upon transplantation. Glasses in region C are chemically unstable as the silica content is low and soda content is high hence degrade quickly when in aqueous environment (< 30 days). Region D contains composition with very low silica content such that it is not possible for it to form a glass network or crystallisation occurs.

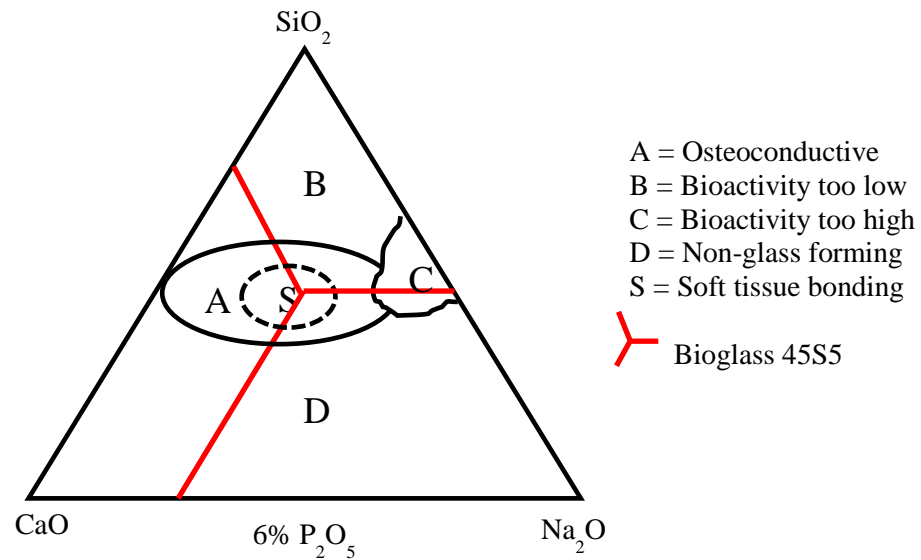


Figure 1.14: Bioactivity map of $\text{SiO}_2 - \text{Na}_2\text{O} - \text{CaO}$ glass system showing regions of bioactivity [105].

Bioactive glasses when immersed in aqueous solutions such as body fluids or simulated body fluids, dissolve slowly and release dissolution products such as calcium, sodium or phosphate ions, which are found within the body, particularly in bone. The dissolution products of bioactive glasses can form a carbonated hydroxyapatite layer on their surface [108, 112] which structurally and chemically is like the mineral phase of bone thus binds firmly with living bone and tissue [102, 112, 113]. Initially bioactive glasses were used as a material for bone regeneration and since the discovery of 45S5, bioactive glasses have been extensively investigated for their use in dental and orthopaedic fields. For instance, monolithic 45S5 was used as middle ear prostheses to restore the ossicular chain and to treat conductive hearing loss. It is also used as a synthetic bone graft to regenerate and heal bone defects [114]. Recently particulate bioactive glass can be found as the active ingredient of toothpaste to reduce sensitivity in teeth [105]. However, in recent years bioactive glasses have been shown to possess useful properties such as angiogenesis, remineralising potential and antimicrobial activity [112].

1.6.4 Antimicrobial properties

The ability of bioactive glasses to kill microorganisms has been investigated in the past two decades. It was not until 1998 that Stoor *et al.*, investigated the antimicrobial effect of particulate bioactive glass on oral microorganisms [115]. *In vitro* S53P4 was shown to kill pathogens connected with enamel caries (*Streptococcus mutans*), root caries (*Actinomyces naeslundii*, *S. mutans*) and periodontitis (*Actinobacillus actinomycetemcomitans*) [116]. The same glass composition demonstrated antimicrobial effect against *E. faecalis* in dentinal tubules [117]. Similarly, 45S5 Bioglass was shown to reduce supragingival and subgingival bacteria [118] as well as persistent root canal infections due to enterococci [119].

The exact mechanism of action of bioactive glasses is not yet clearly understood, however, initial studies on the antimicrobial efficacy attributed the killing of microbes due to changes in the pH of the solution. Like all cells, microorganism can tolerate changes in pH within an optimum range, however a significant change in the pH is detrimental to cells. According to Hu *et al.* [120], the alkali ions released from the bioactive glass alters the pH gradient of the cytoplasmic membrane which is required for the movement of organic nutrients and components into the cell. A higher, i.e. non-physiological concentration of dissolution products effects the osmotic pressure and thus cause cell death. While silica based bioglasses have shown some antimicrobial properties, the associated limitations such cytotoxic effects of prolonged silica exposure and slow degradation has led to the emergence of phosphate based glasses as potential alternatives [121].

1.7 Phosphate glasses

Glasses can be categorised based on the glass formers, those with phosphate as the network former are termed phosphate glasses. As discussed in section 1.6, silicate glasses have been extensively researched for many years for their tissue regeneration properties [121]. Many silicate glasses including 45S5 have shown success in dental and orthopaedic applications, however the long-term use of these glasses has been questioned due to their slow degradation *in vivo* which

can take up to two years [122, 123]. Due to this limitation, the search for new biomaterials has led to the emergence of phosphate glasses.

Like silicate glasses, the building block of the phosphate glasses is a tetrahedral unit. The glass forming component is P_2O_5 and the basic unit is the orthophosphate (PO_4^{3-}) tetrahedron (a phosphorus atom surrounded by four oxygen atoms). The PO_4 unit is different from the SiO_4 unit as phosphorus has a 5+ charge where one of the oxygen atoms share their two-unpaired electron with the P^{5+} ions and form a terminal double bond whereas silicon has 4+ where all oxygen atoms form single bonds (Figure 1.15). Whilst in a silica tetrahedral unit all oxygen atoms are equally shared and act as bridges, in phosphate tetrahedron only three oxygen atoms act as bridges, which limits the connectivity of phosphate glasses relative to their silicate counterparts [121]. This makes phosphate glasses less rigid or durable compared to silicate glasses [124].



Figure 1.15: Silicate and Phosphate tetrahedral structures.

Unlike pure vitreous silica which is chemically and thermally stable, pure P_2O_5 glasses are very reactive and hygroscopic therefore modifiers such as calcium and sodium oxide are added to improve their durability [105]. Like all other bioactive glasses, the bioactivity of phosphate glasses is determined by their structure, i.e. content of P_2O_5 and network modifiers. Unlike silicate glasses, vitreous P_2O_5 is susceptible to hydrolysis as water or atmospheric humidity can disrupt the glass structure by creating POH groups instead of POP linkages [105].

Phosphate glasses belong to a class of materials known as controlled release glasses (CRG) which have several distinctive properties making them a unique group of materials. The most desirable characteristic is their complete dissolution in aqueous solutions, leaving no residues. The dissolution rate can be altered from hours to several weeks by adjusting the glass composition and thus a wide range of glasses can be prepared according to the end application. Not only does their dissolution remain constant for as long as the material remains in the body but also the components of the glass can be easily excreted as these are found naturally in human body [121]. The ability of phosphate glasses to dissolve at a constant rate and the non-toxic nature of the dissolution products makes them highly favourable for clinical applications. Like any bioactive glass the immersion of these glasses in aqueous solution results in glass degradation, as the glass degrades it affects not only the ionic composition of the environment but also the pH. Generally, with an increasing P_2O_5 content the pH decreases and vice versa.

As stated earlier, the dissolution of glasses results in release of ions in the local environment, therefore biologically active elements such as metal ions can be incorporated and delivered at constant rate into surrounding aqueous environment. Phosphate glasses doped with antimicrobial ions can be used as localised antimicrobial delivery systems [121]. Whilst studies [125, 126] have shown the antimicrobial effect of phosphate glasses with only calcium and sodium as modifiers, the inclusion of antimicrobial ions such as copper, silver and gallium have shown additional benefits in killing microorganisms[127-129].

In recent years phosphate-based glasses have been prepared by incorporating antimicrobial components such as silver and copper for dental applications. Knowles *et al.* [130] investigated the antimicrobial effect of silver-doped phosphate-based glass on planktonic bacteria as well as the effect of increasing copper content in phosphate based glasses on biofilms of *Streptococcus sanguinis* [127]. Both studies demonstrated antimicrobial properties of the given glass compositions. 3 and 5% mol Ag significantly inhibited growth of both Gram positive and negative

bacteria as well as *C. albicans*. 10% and 15% Cu doped glasses also showed a considerable log reduction in bacterial count within 24 hours compared to the glass without copper ions.

When considering bone tissue regeneration, antimicrobial efficacy or any other biological effects of bioactive glasses, it is essential to evaluate the cytotoxic effect of the glasses as well. Bioactive glasses have been extensively investigated for bone repair and regeneration as well as in dental field. Biocompatibility of bioactive glasses is a key element when it comes to their *in vivo* applications. The composition of bioactive glasses has a significant effect on their ability to support cell growth and proliferation. Phosphate glasses or bioactive glasses in general are believed to have non-cytotoxic effects on various cells [112, 131-133].

To conclude, the higher incidence of CA-UTIs and unsuccessful clinical trials of currently available antimicrobial catheters highlights the need to investigate novel antimicrobial coatings for reducing the risk of infections caused by indwelling catheters. Previously glasses have been shown to be safe for inserting into bladder lining without causing major toxic and inflammatory responses [134]. Since the safety of Bioglass has been tested *in vivo* and *in vitro* therefore it can serve as a potential delivery system.

1.8 Aims of the study

The aims of the current project were/are to

- Manufacture and study the dissolution behaviour of 1, 3, 5 and 10 mol % cobalt phosphate glasses
 - Determine the changes in the pH and ionic concentration of the aqueous environment studied
- Manufacture and study the dissolution behaviour of 1, 3, 5 and 10 mol % zinc phosphate glasses
 - Determine the changes in the pH and ionic concentration of the aqueous environment studied
- Determine the antimicrobial properties of 1, 3, 5 and 10 mol % cobalt phosphate glasses against microorganisms in planktonic growth mode.
- Determine the antimicrobial properties of 1, 3, 5 and 10 mol % zinc phosphate glasses against microorganisms in planktonic growth mode.
- Study the antimicrobial efficacy of 5 mol % copper, cobalt and zinc doped phosphate based glasses; alone and in combination against microorganisms in planktonic growth mode.
- Undertake a cytotoxic evaluation of 3, 5 and 10 mol % cobalt doped phosphate glasses against uroepithelial cells
- Undertake a cytotoxic evaluation of 3, 5 and 10 mol % zinc doped phosphate glasses against uroepithelial cells.

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Chapter 2

Manufacturing and study of dissolution behaviour of cobalt and zinc doped phosphate glasses

2.1 Introduction

Phosphate glasses, due to lack of chemical stability, are soluble in aqueous media and thus can be used for a wide range of biomedical applications. The highly favoured characteristic of these glasses is the controlled release of the ions in to the surrounding media without toxic effects. Phosphate based glasses have long been in existence however, their application was limited by their lack of chemical durability. In the 1950s, the addition of 30% CaO to pure vitreous P_2O_5 was shown to significantly improve the glass durability [1]. It is now well established that the rate of dissolution of phosphate glasses can be controlled by varying the composition of the glasses. In the past few decades these glasses have been widely studied as controlled release glasses that can be used to deliver specific ions in a local environment. Phosphate based glasses have uniform dissolution over a period within ten minutes of leaching and the system is believed to maintain the dissolution for over a year [2]. It has also been demonstrated that the dissolution is constant and non-selective leaching occurs.

As previously stated, phosphate based glasses consist of PO_4 tetrahedron which is connected to a maximum of three neighbouring tetrahedra forming a network. Addition of modifiers such as sodium or calcium leads to depolymerisation of the network and creates non-bridging oxygen atoms. The mechanical strength of the glass is thus increased due to the ionic cross-links between non-bridging oxygen atoms. Several theories have been proposed to elucidate and understand the dissolution mechanism of phosphate based glasses. According to the widely accepted theory, proposed by Bunker *et al.* [2] the glass dissolution occurs in two interdependent steps. In the first step a hydrated layer is formed on the surface of the glass due to exchange of sodium ions in glass with hydrogen ions in water, Figure 2.1 [2-4].



Figure 2.1: showing the dissolution mechanism of phosphate glasses; adapted from Gao *et al.*[3].

This step results in leaching of alkali or alkaline earth metals into water creating a P_2O_5 rich surface layer. In the second step the P-O-P bonds in the hydrated layer breaks and release phosphate chains of varying length into the solution [3]. However contrary to this theory Delahaye *et al.*, argued that the dissolution of the glasses is controlled by ionic strength of the solution and not the ion exchange [5]. Throughout the whole process the phosphate glass dissolves congruently which means that the dissolution products that are in the solution have identical composition with that of the bulk glass [6]

The solubility of phosphate glasses can be tailored by changing the compositions i.e. by the addition of various metal oxides. For instance, the addition of alkali oxides to phosphate glasses has been shown to increase the dissolution rate in aqueous environment whereas addition of alkaline earth metals increases the chemical stability. For this study, a ternary glass P_2O_5 - Na_2O - CaO was selected as the delivery system. These glasses are not only biocompatible with low

toxicity and inflammatory response but also their solubility can be tailored by varying the amount of calcium oxide. An increase in CaO content decreases the glass solubility and vice versa [7-9]. Since the solubility of these glasses is linear with time therefore incorporating antimicrobials in such glass system will ensure constant delivery of the active ions as the glass dissolves. In the given study dissolution behaviour of sodium calcium phosphate glasses doped with cobalt and zinc was investigated.

2.2 Aims

The aim of the given study was to manufacture cobalt and zinc doped phosphate based glasses and investigate various parameters associated with glass dissolution. This was achieved by studying:

- Dissolution behaviour of glasses with increasing metal oxide content
- Degradation rates of various glass compositions in different aqueous media such as distilled water, nutrient broth, synthetic urine and cell culture media
- Changes in pH values of the surrounding media when glasses dissolve
- The release of ionic species via inductively coupled plasma analysis as a function of time.

2.3 Methods and Materials

2.3.1 Glass preparation

The glasses were prepared using a melt quench method as the technique is simple, low cost and requires less time to complete compared to sol-gel glasses. The starting precursors were P_2O_5 (99%, Fisher Scientific), NaH_2PO_4 (>99.0%, Sigma Aldrich, Dorset, UK) and $CaCO_3$ (99.95%, Alfa Aesar, Lancashire, UK). To produce cobalt, copper and zinc containing phosphate based glasses CoO (99%, Sigma Aldrich, Dorset, UK), $CuSO_4$ (99%, Sigma Aldrich, Dorset, UK) and ZnO (99%, Fisher Scientific) was used respectively. The glass prepared had a fixed concentration of P_2O_5 (50 mol %) along with a fixed Na_2O content of 20 mol %. The remaining CaO (30-x) was substituted accordingly with X amount of CoO , $CuSO_4$ or ZnO . The glass compositions investigated are given in the Tables 2.1a and 2.1b below:

Mol %				
	P_2O_5	CaO	Na_2O	CoO
0% mol Co	50	30	20	0
1% mol Co	50	29	20	1
3% mol Co	50	27	20	3
5% mol Co	50	25	20	5
10% mol Co	50	20	20	10

Table 2.1a: Compositions of cobalt doped phosphate glasses investigated.

	Mol %			
	P ₂ O ₅	CaO	Na ₂ O	ZnO
0% mol Zn	50	30	20	0
1% mol Zn	50	29	20	1
3% mol Zn	50	27	20	3
5% mol Zn	50	25	20	5
10% mol Zn	50	20	20	10

Table 2.1b: Compositions of zinc doped phosphate glasses investigated

To evaluate the effect of incorporating two different metal oxides in one glass, additional samples were prepared. These glasses had a fixed concentration of P₂O₅ (50 mol %) along with a fixed CaO and Na₂O content, 20 mol % each. The remaining 10% was substituted accordingly with 5 mol % of CoO, ZnO or CuO. The combinations investigated were Co/Zn, Co/Cu and Cu/Zn; as shown in the Table 2.2

	Mol %					
	P ₂ O ₅	CaO	Na ₂ O	CoO	CuO	ZnO
Co/Cu	50	20	20	5	5	0
Co/Zn	50	20	20	5	0	5
Cu/Zn	50	20	20	0	5	5

Table 2.2: Glass compositions for evaluating synergistic potential of the glasses

The required chemicals were weighed out and placed into a 59 ml Pt - 10% Rh crucible (GLC alloys Ltd Middlesex, UK). After mixing the chemicals thoroughly the crucible was placed in a furnace at room temperature. The furnace was heated up to 300°C (10°C per minute) and dwelled for an hour. The temperature was then raised up to 600°C (60°C per minute) and after dwelling for 30 minutes, once again temperature was rapidly increased (60°C per minute) up to 1050°C and held at this temperature for 30 minutes. The molten glass was then poured into a split graphite mould, with 10 mm aperture (Figure 2.2), which had been preheated at 350°C.

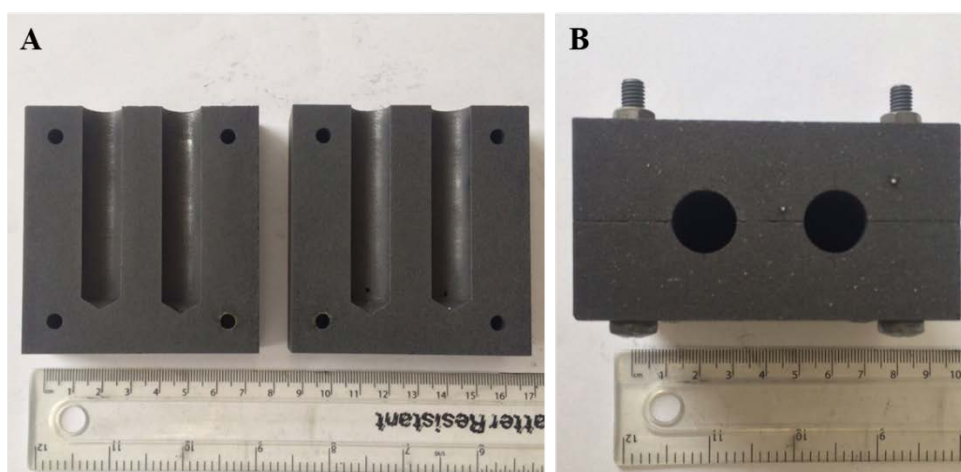


Figure 2.2: A split graphite mould (A – open, B – closed) with 10 mm aperture.

The glass samples were allowed to slowly cool down to room temperature (inside furnace previously heated to 350°C) and the resulting glass rods were cut into 2.5 mm thick discs with an IsoMet™ 1000 Precision saw using Cool 2 cutting fluid as lubricant (Buehler). Discs were polished using MetaServ® (Buehler) polishing machine using a series of polishing cloths (30µ, 15µ using lapping oil) followed by a final polish using 0.02µ colloidal silica. The discs were cleaned in acetone and air dried. To determine the effect of glasses in combinations, glass powders were used. Each glass composition was ground using mortar and pestle and sieved in the particle size range from 40-60 microns. Prior to undertaking the experiments, glass discs and powders

were sterilised using dry heat at 180°C for 2 hours. Samples were stored in a desiccator between stages of preparation to reduce exposure to atmospheric moisture.

2.3.2 Glass characterisation using High Energy X-ray Diffraction

High energy X-ray diffraction was carried out to determine the amorphous nature of the manufactured glasses. The experiments were carried out at I-15 beamline at Diamond Light Source, Harwell, UK. The instrument was set up to collect data in a 2 θ geometry with a Si monochromatic, the energy of the X-ray beam was 76.7 KeV, $\lambda = 0.162$ Å. Finely ground glass powders were loaded in to 1.17 mm x 1.5 x 40 mm SiO₂ glass capillaries at room temperature and mounted on sample changer (placed at right angles to incident X-ray beam). An empty capillary was measured to account for background corrections. Data corrections and normalizations were carried out using GudrunX [10].

Degradation studies

Glass rods with 10 mm diameter were cut into 2.5 mm thick discs and polished to 400 grit finish. To determine the surface area of the discs their exact thickness was measured using Vernier callipers (Fisher Scientific Loughborough, Leicestershire). The discs were then placed in 60 ml plastic containers with an internal diameter of 37 mm (Fisher Scientific Loughborough, Leicestershire) filled with 25 ml of standard distilled water (SDW). The containers were then placed on an orbital shaker in a 37°C incubator at 200 rpm. At various time intervals, i.e. every 24 hours for 7 days glass discs were taken out of their respective containers and weighed after removing the excess moisture using paper towel. To determine the degradation rate, weight loss was calculated, as shown below.

$$\text{Weight loss} = (M_0 - M_t)/A \quad \dots\dots\dots (2.1)$$

where, M_0 – initial weight in mg

M_t – weight at time t in mg

A is the surface area, cm^2

Weight loss was plotted against time and the slope of the graph gave a degradation value ($\text{mg cm}^{-2} \text{ h}^{-1}$). Degradation analysis was also conducted in nutrient broth (NB), Surine™ (SU- synthetic urine) and in urothelial cell culture media (CM) supplemented with 10% FBS and 1% penicillin/streptomycin. Nutrient broth, Surine™ and Urothelial cell media were purchased from Oxoid, Sigma Aldrich and ScienCell laboratories respectively. Each measurement was carried out in triplicate.

2.3.3 pH analysis

pH measurements of the degradation media were taken at every time point using pH meter (Mettler Toledo, Switzerland). Three-point calibration of the pH electrode was achieved using pH calibration standards; 4.0, 7.0 and 10 (Fisher Scientific, Loughborough, UK) at 25°C.

2.3.4 Ion release study

The medium obtained at each time point from degradation studies was analysed for the presence of cation (Na^+ , Ca^{2+} , Co^{2+}) and anion (PO_4^{3-}) using inductively coupled plasma optical emission spectrometry, ICP-OES (iCAP™ 7000 Plus Series). ICP-OES is an analytical technique that is used for quantification of trace metal ions in a sample. Three ml of each samples was initially passed through a nebuliser to create aerosols which are then passed through inductively coupled plasma at high temperatures to produce excited ions. The resulting species were then separated and detected with an optical emission spectrometer (OES). The dissolution products were filtered prior to measurements using 0.2 μm Minisart filters (Fisher Scientific, UK) and the concentration of each ion was calculated from the linear portion of the generated standard curve as ppm. A

standard curve is the relationship between instrument response and known concentration in the analyte. The curves were produced using a blank and a five-point calibration (0.1, 0.5, 1.0, 5.0 and 10.0 ppm) of the elements investigated in the study. The calibration samples along with quality control samples were diluted in 1 N nitric acid.

2.4 Results

2.4.1 Cobalt doped phosphate glasses

2.4.1.1 Glass characterisation using High Energy X-ray Diffraction

The amorphicity of the cobalt doped phosphate glasses was determined by using high energy X-ray diffraction analysis. Figure 2.3 shows data from the phosphate based glasses doped with 1, 3, 5 and 10 mol% cobalt doped along with an un-doped glass. Crystalline solids reveal significant and sharp peak when characterised whereas the broad peaks are indicative of amorphous structure. The HEXRD pattern shows broad peaks suggesting that the glasses obtained were amorphous.

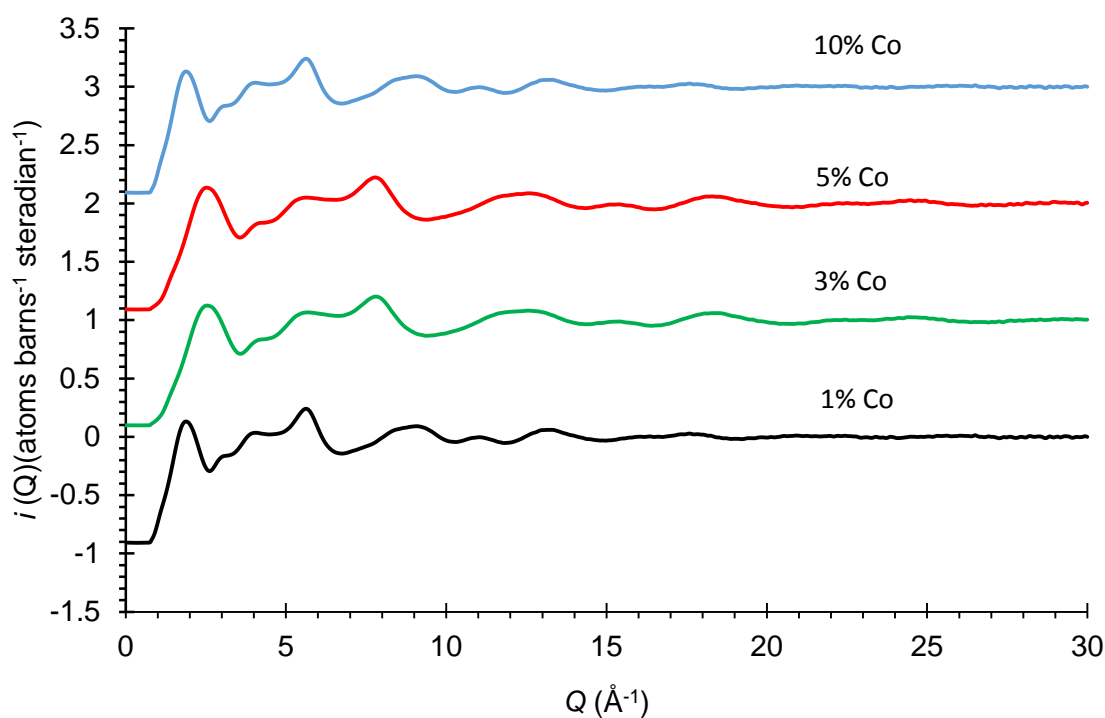
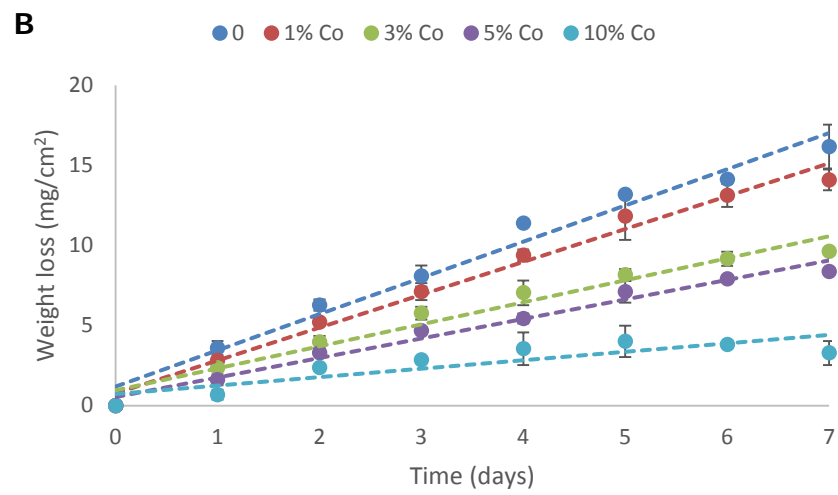
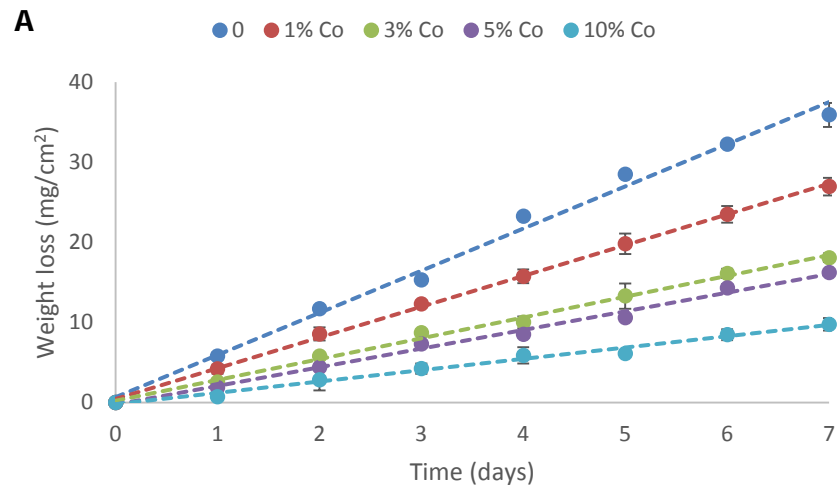


Figure 2.3: High Energy X-ray diffraction pattern of synthesised cobalt doped phosphate based glasses (1, 3, 5 and 10 mol %) measured at I-15 beamline at Diamond Light Source, Harwell, UK.

2.4.1.2 Degradation analysis

Degradation data for each glass composition, shown in Figure 2.4, was obtained by applying a line of best fit through the weight loss per unit area against time. A linear increase in weight loss as a function of time is observed for all samples in all solutions. The control glass (un-doped phosphate glass) has the largest weight loss and this weight loss decreases with increasing CoO content for all the solutions tested. Nutrient broth and cell culture media displayed broadly similar dissolution profiles of $\sim 2.3 - 2.6 \text{ mg/cm}^2$ for the control glass, with the dissolution reducing by an order of magnitude for the 10% doped CoO glass. Values of 2.1, 1.4 and 1.1 mg/cm^2 were observed for the 1%, 3% and 5% CoO respectively.

Whilst the dissolution of glasses in all media followed the same trend of decrease in dissolution with an increase in cobalt oxide content, the rate of degradation was however significantly different. The dissolution rates were approximately twice as high when the samples were placed in distilled water compared to nutrient broth and cell culture media. For instance, the degradation value at day 7 for 1% cobalt glass in distilled water was 27 mg/cm^2 whereas the degradation rate was 14 mg/cm^2 and 15 mg/cm^2 in nutrients broth and cell culture media respectively. Glass degradation was significantly higher when immersed in synthetic urine, for example the control and 1% CoO glasses weight loss was ~ 7.5 times greater in synthetic urine compared to nutrient broth and cell culture media.



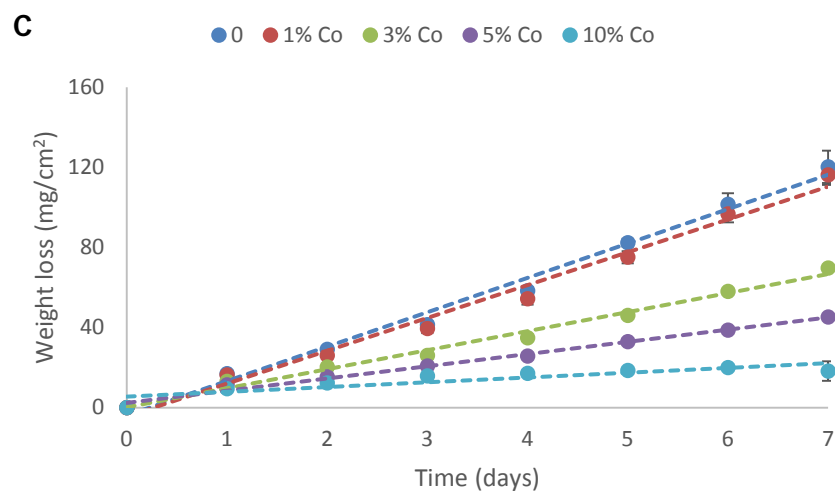
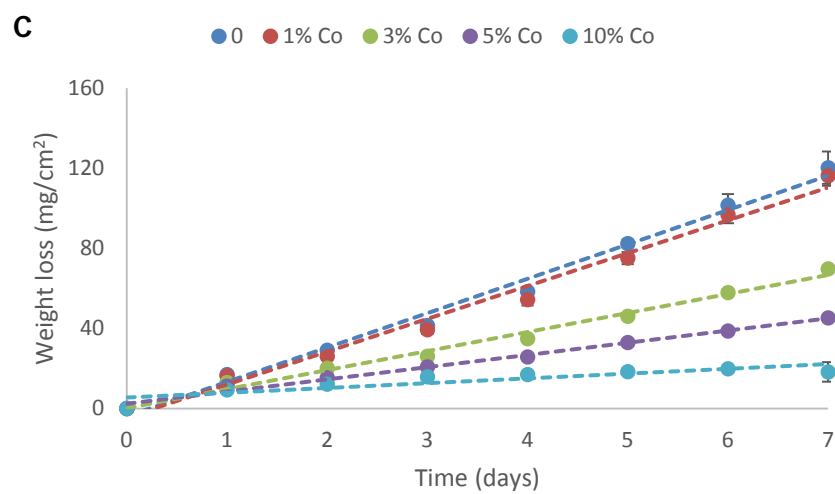


Figure 2.4: The degradation profiles obtained for 0, 1, 3, 5 and 10 mol % Co compositions, investigated in distilled water (A), nutrient broth (B), synthetic urine (C) and cell culture media (D). The data shows variable degradation rate in different media. (Error bars = \pm SD for triplicate samples).

2.4.1.3 pH studies

The pH analysis displayed a sharp decrease in the pH value of the distilled water for all compositions within 24 hours where the values dropped from 6.7 to ~ 3.9 - 4.5 for the remaining duration of the study, as seen in the Table 2.1. Cobalt free composition showed the maximum decrease in the pH value followed by 1 mol % cobalt glass. The pH values observed for 3 and 5 mol % cobalt glasses were similar from day 0 to 7. 10 mol % cobalt compositions however had slightly higher pH values than 3 and 5 mol % compositions.

On the other hand, nutrient broth, cell culture media and synthetic urine appeared to have a buffering capacity as the pH values remained within a narrow range. The pH of nutrient broth and cell culture media were in the optimum range for the duration of the study between 6.3-6.9 and 7.8-8.3. However, the pH values in synthetic urine was highly alkaline due to the initial alkaline pH of the synthetic urine.

	Distilled water					Nutrient broth				
Day	0	1%	3%	5%	10%	0	1%	3%	5%	10%
0	6.72±0.0	6.72±0.0	6.72±0.0	6.72±0.0	6.72±0.0	6.5±0.0	6.5±0.0	6.5±0.0	6.5±0.0	6.5±0.0
1	5.13±0.22	4.72±0.05	4.8±0.02	4.85±0.02	5.04±0.02	6.84±0.04	6.82±0.0	6.93±0.04	6.93±0.04	6.88±0.03
2	4.56±0.02	4.44±0.14	4.46±0.01	4.48±0.01	4.6±0.02	6.78±0.01	6.78±0.01	6.78±0.01	6.78±0.01	6.89±0.01
3	4.15±0.09	4.20±0.09	4.31±0.04	4.32±0.06	4.54±0.03	6.25±0.01	6.70±0.01	6.73±0.04	6.73±0.01	6.77±0.02
4	4.18±0.03	4.20±0.01	4.35±0.01	4.37±0.02	4.55±0.21	6.48±0.0	6.57±0.04	6.69±0.04	6.69±0.02	6.73±0.01
5	4.16±0.04	4.15±0.014	4.25±0.03	4.29±0.01	4.50±0.03	6.72±0.01	6.42±0.06	6.5±0.02	6.5±0.01	6.71±0.11
6	3.93±.007	4.02±0.05	4.12±0.02	4.08±0.01	4.21±0.04	6.48±0.0	6.56±0.05	6.69±0.04	6.69±0.02	6.73±0.014
7	3.93±0.014	3.95±0.05	4.10±0.02	4.02±0.01	4.10±0.01	6.28±0.0	6.30±0.01	6.45±0.01	6.48±0.02	6.73±0.19

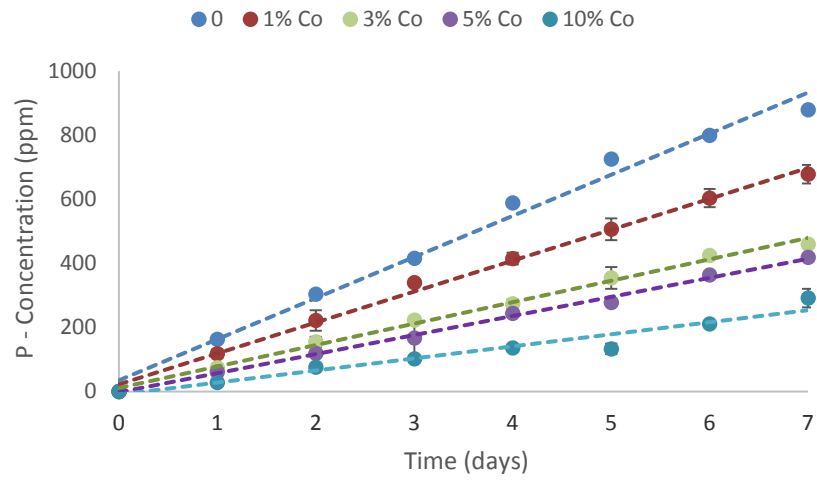
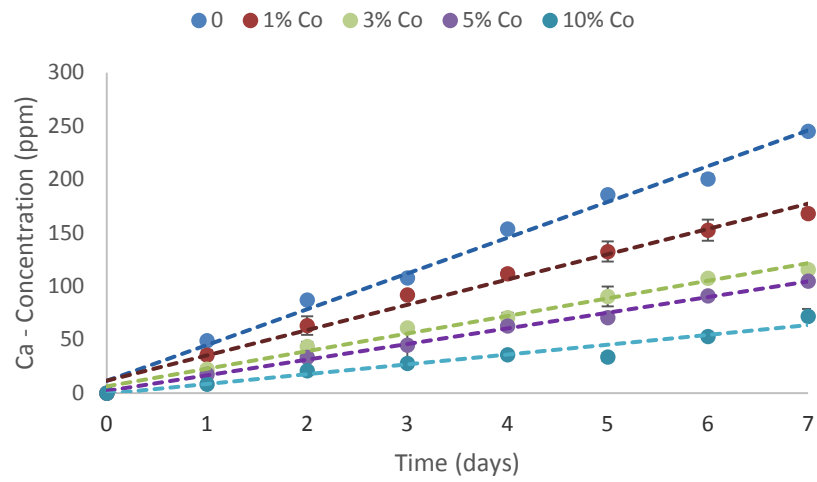
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	Synthetic urine					Cell Culture media				
Day	0	1%	3%	5%	10%	0	1%	3%	5%	10%
0	9.54±0.0	9.54±0.0	9.54±0.0	9.54±0.0	9.54±0.0	7.86±0.0	7.86±0.0	7.86±0.0	7.86±0.0	7.86±0.0
1	9.48±0.01	9.47±0.04	9.54±0.01	9.44±0.01	9.54±0.01	7.84±0.0	7.91±0.0	7.94±0.0	7.9±0.0	7.87±0.0
2	9.37±0.02	9.38±0.02	9.47±0.03	9.49±0.01	9.54±0.01	7.90±0.0	7.91±.0	7.85±0.0	7.85±0.0	8.02±0.0
3	9.27±0.03	9.25±0.02	9.43±0.02	9.47±0.03	9.50±0.01	7.89±0.0	7.93±0	7.85±0.0	7.85±0.0	8.09±0.0
4	9.20±0.02	9.19±0.03	9.33±0.03	9.39±0.01	9.41±0.01	7.69±0.0	7.74±0.0	7.92±0.0	7.92±0.0	8.06±0.0
5	9.12±0.01	9.17±0.02	9.23±0.02	9.31±0.02	9.37±0.01	7.72±0.0	7.81±0.0	7.96±0.0	7.96±0.0	8.12±0.0
6	9.06±0.00	9.09±0.03	9.14±0.03	9.25±0.01	9.31±0.01	7.61±0.0	7.72±0.0	7.88±0.0	7.88±0.0	8.19±0.0
7	9.10±0.03	9.17±0.01	9.24±0.01	9.26±0.01	9.32±0.01	7.73±0.0	7.83±0.0	7.98±0.0	7.98±0.0	8.34±0.0

Table 2.2: shows the pH values of cobalt doped glasses (0, 1, 3, 5 and 10 mol % Co compositions), investigated in distilled water, nutrient broth, synthetic urine and cell culture media (Mean ± SD for triplicate sample).

2.4.1.4 Ion release study (ICP-OES)

Figure 2.5 shows the cumulative ion release profile of cobalt doped phosphate glasses in distilled water. The data correlated with the weight loss profiles of the glass compositions. The study showed the highest anion (PO_4^{3-}) and cation (Ca^{+2} and Na^{+}) release for the compositions with the highest dissolution rate, i.e. the cobalt free composition released the greatest number of ions and cobalt doped glasses ion release profile was in the order $1 > 3 > 5 > 10$ mol %. The result showed that the rate of cobalt ion release is also correlated to the rate of degradation with a considerable difference between the 1, 3 and 5 mol % compositions, however no substantial difference was detected between 5 and 10 mol % cobalt glasses.

A**B**

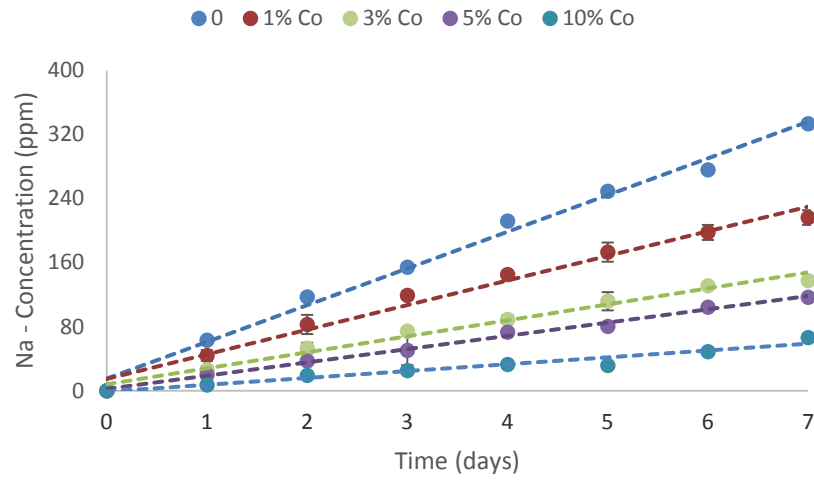
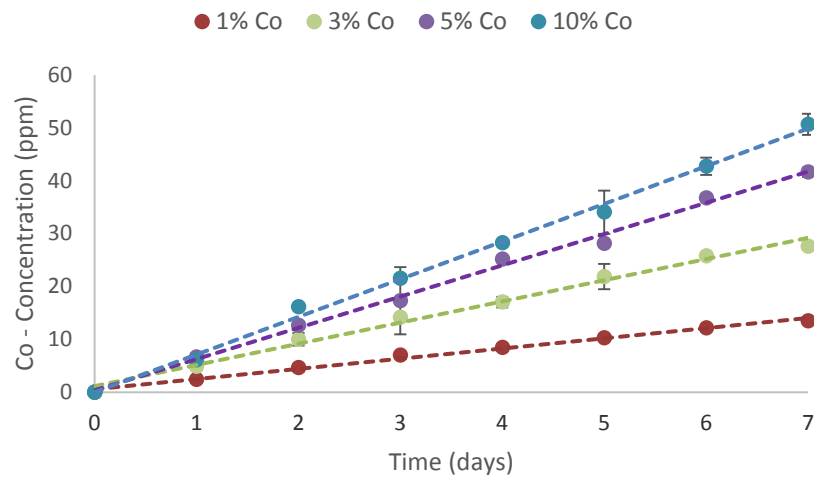
C**D**

Figure 2.5: Cumulative ion release (A) phosphorus, (B) calcium, (C) sodium and (D) cobalt as a function of time for 0, 1, 3, 5 and 10 mol% cobalt doped glasses in distilled water. (Error bars = \pm SD for triplicate samples).

2.4.2 Zinc doped phosphate glasses

2.4.2.1 Glass characterisation using High Energy X-ray Diffraction

High energy X – ray diffraction was performed to confirm the amorphicity of zinc doped phosphate glasses. Figure 2.6 shows data from the phosphate based glasses doped with 1, 3, 5 and 10 mol% zinc doped along with an un-doped glass. Crystalline solids reveal significant and sharp peak when characterised whereas the broad peaks shown in Figure 2.6 suggest that the glasses obtained were amorphous.

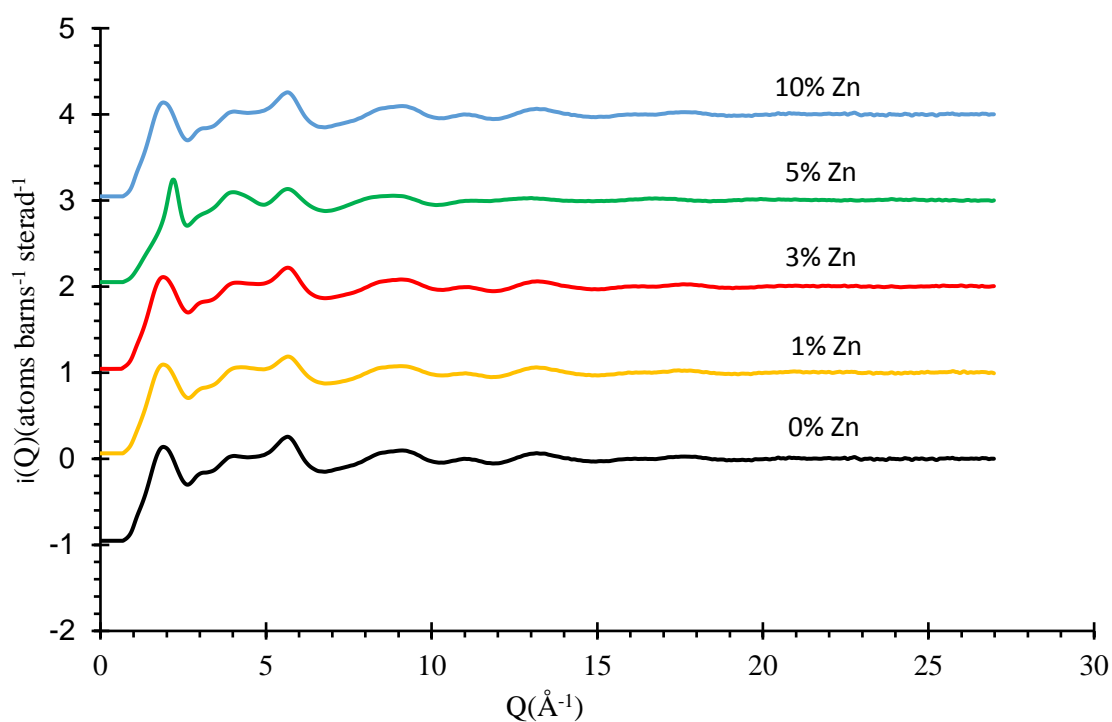
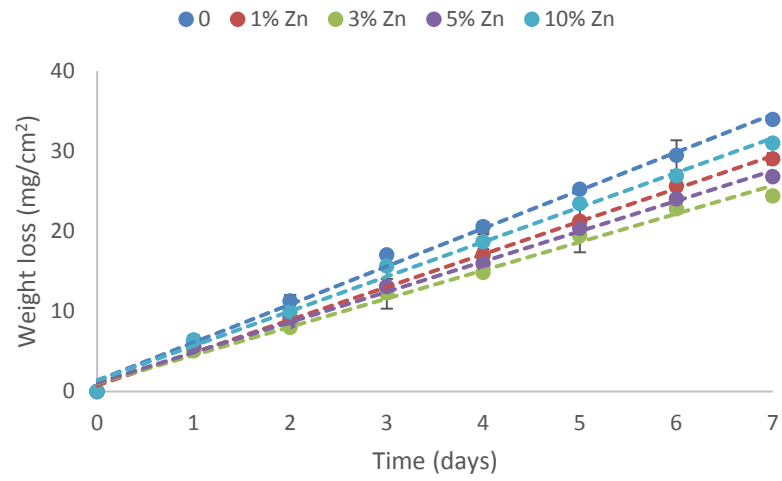


Figure 2.6: High energy x ray diffraction data on zinc doped phosphate based glasses (0, 1, 3, 5 and 10 mol %) measured at I-15 beamline at Diamond Light Source, Harwell, UK.

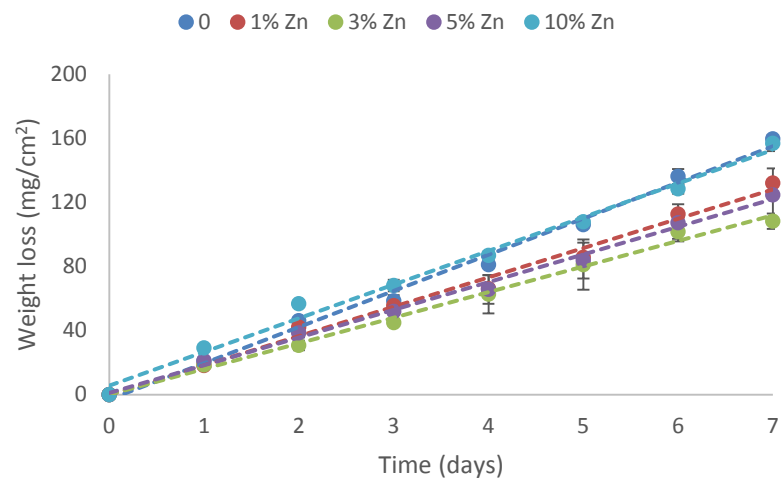
2.4.2.2 Degradation analysis

Figure 2.7 shows weight loss per unit area measured (mg/cm^2) over a week. Unlike cobalt doped glasses where the weight loss was observed to decrease with an increasing cobalt oxide content, zinc also showed a mixed dissolution behaviour. Glass degradation decreased with an increasing zinc oxide content up to 3 mol % Zn, however an increase in dissolution/weight loss was observed for 5 mol % Zn and 10 mol % Zn compared to 3 mol % Zn. The order of weight loss observed was $0 > 1 > 3 < 5 < 10$ mol %. The weight loss for 10 mol % Zn was similar to zinc free composition, for example at day 7 the weight loss for zinc free glass was $33 \text{ mg}/\text{cm}^2$ and $31 \text{ mg}/\text{cm}^2$ for 10 mol % Zn glass, whereas 3 mol % Zn was least soluble composition ($24 \text{ mg}/\text{cm}^2$) of the glasses analysed. The dissolution trend described above was also observed in other media; nutrients broth, synthetic urine and cell culture media (Figure 2.7) however, the cumulative mass loss observed in nutrient broth and cell culture media was almost half that of distilled water and four times that in synthetic urine.

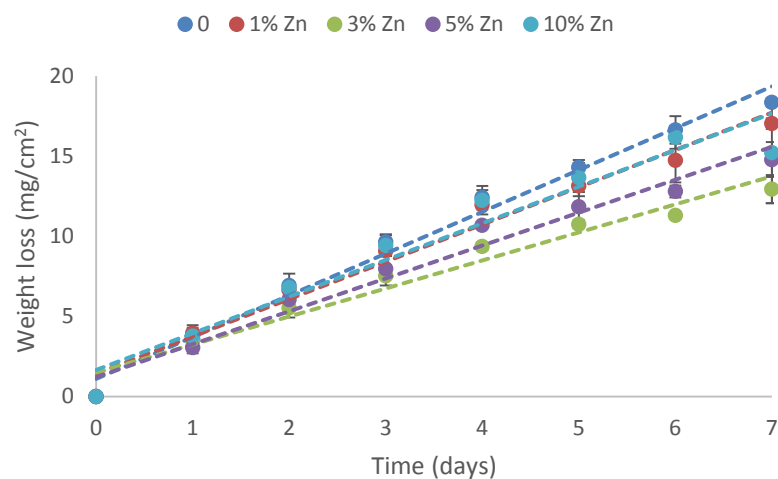
A



B



C



D

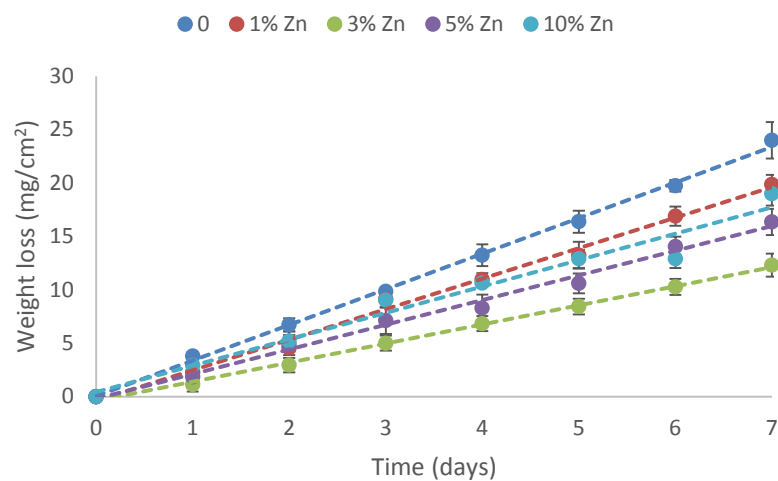


Figure 2.7: The degradation profiles obtained for 0, 3, 5 and 10 mol % Zn compositions, investigated in distilled water (A), nutrient broth (B), synthetic urine (C) and cell culture media (D). The data shows variable degradation rate in different media. (Error bars = \pm SD for triplicate samples).

2.4.2.3 pH studies

The pH values of the zinc doped glasses in different media are given in Table 2.3. The pH values of the zinc free glass composition, in distilled water, decreased sharply in the first 24 hours. A gradual increase was seen in the remaining period with the lowest value 3.8 on day 7 of the analysis. Similarly, the pH of the zinc oxide composition decreased rapidly within 24 hours falling to between 5.6 and 5.7. The pH of the zinc containing glasses did not decrease as much as that of the zinc free composition by day 7.

On the other hand, a negligible change in the pH was observed for the glasses in nutrient broth, synthetic urine and cell culture media. The pH values remained in the optimum range throughout the study for nutrient broth (6.30 - 6.97) and cell culture media (7.41 - 7.56), however due to the initial alkaline pH of synthetic urine (9.72) high pH values were observed. The results obtained were in agreement with the pH studies of cobalt doped glasses (section 2.4.1.3)

	Distilled water					Nutrient broth				
Day	0	1%	3%	5%	10%	0	1%	3%	5%	10%
0	7.37±0.0	7.37±0.0	7.37±0.00	7.37±0.0	7.37±0.0	6.97±0.0	6.97±0.0	6.97±0.0	6.97±0.0	6.97±0.0
1	5.24±0.19	5.74±0.10	5.70±0.03	5.56±0.0	5.47±0.30	6.94±0.01	6.86±0.02	6.81±0.01	6.79±0.0	6.81±0.02
2	4.78±0.01	5.16±0.0	5.41±0.07	5.36±0.07	5.42±0.04	6.7±0.01	6.77±0.02	6.78±0.01	6.77±0.01	6.52±0.01
3	4.20±0.19	4.58±0.27	4.62±0.02	4.61±0.03	4.52±0.26	6.69±0.0	6.69±0.0	6.60±0.0	6.55±0.0	6.41±0.0
4	4.23±0.01	4.47±0.11	4.50±0.05	4.42±0.09	4.47±0.16	6.48±0.0	6.56±0.04	6.69±0.04	6.70±0.02	6.51±0.01
5	3.96±0.04	4.28±0.12	4.31±0.02	4.25±0.02	4.185±0.02	6.57±0.06	6.41±0.06	6.50±0.02	6.48±0.01	6.51±0.11
6	3.96±0.16	4.29±0.02	4.25±0.0	4.21±0.01	4.08±0.01	6.48±0.0	6.56±0.04	6.69±0.04	6.70±0.02	6.53±0.01
7	3.80±0.07	4.23±0.09	4.28±0.04	4.14±0.02	4.04±0.01	6.28±0.0	6.3±0.01	6.48±0.01	6.48±0.02	6.38±0.08

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	Synthetic urine					Cell Culture media				
Day	0	1%	3%	5%	10%	0	1%	3%	5%	10%
0	9.72±0.0	9.72±0.0	9.72±0.0	9.72±0.0	9.72±0.0	7.56±0.02	7.56±0.02	7.56±0.02	7.56±0.02	7.56±0.02
1	9.80±0.01	9.82±0.0	9.65±0.14	9.86±0.04	9.72±0.01	7.51±0.03	7.52±0.0	7.55±0.01	7.55±0.01	7.55±0.01
2	9.82±0.03	9.88±0.04	9.66±0.12	9.71±0.09	9.71±0.04	7.55±0.01	7.52±0.02	7.51±0.03	7.56±0.04	7.54±0.04
3	9.88±0.02	9.79±0.01	9.78±0.06	9.70±0.05	9.73±0.03	7.49±0.02	7.55±0.04	7.47±0.02	7.55±0.06	7.60±0.02
4	9.72±0.06	9.73±0.05	9.71±0.05	9.71±0.02	9.70±0.05	7.47±0.04	7.58±0.06	7.58±0.06	7.51±0.03	7.54±0.01
5	9.77±0.02	9.71±0.06	9.66±0.09	9.68±0.05	9.74±0.01	7.48±0.02	7.50±0.05	7.49±0.07	7.49±0.07	7.51±0.07
6	9.51±0.09	9.79±0.05	9.72±0.05	9.61±0.07	9.69±0.03	7.45±0.06	7.47±0.04	7.51±0.01	7.47±0.06	7.49±0.05
7	9.55±0.14	9.66±0.12	9.81±0.01	9.65±0.11	9.72±0.02	7.40±0.05	7.48±0.07	7.55±0.06	7.41±0.03	7.49±0.02

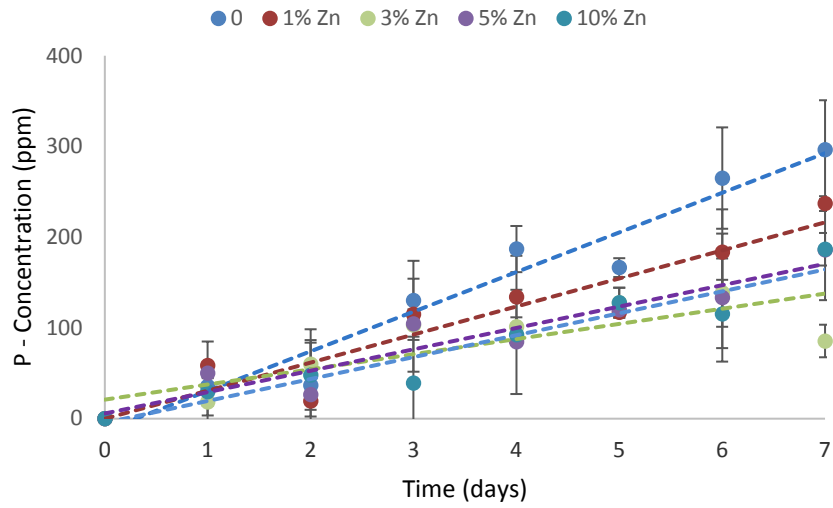
Table 2.3: shows the pH values of zinc doped glasses (0, 3, 5 and 10 mol %), studied in distilled water, nutrient broth, synthetic urine and cell culture media (Mean ± SD for triplicate sample).

2.4.2.4 Ion release study (ICP-OES)

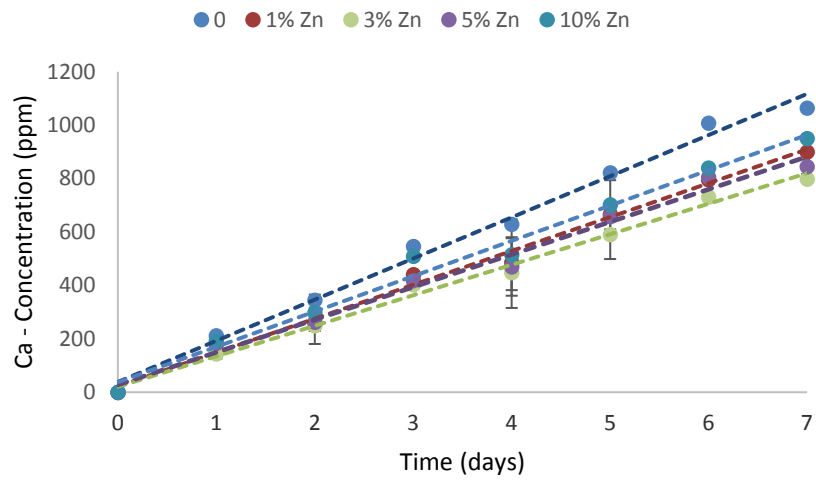
The ion release profile was conducted to monitor the release of cations and anions from the different glass compositions in water over the given period (Figure 2.8). The ion release reflected the degradation behaviour of the glass compositions. A linear increase in the release of anion (PO_4^{3-}) from all compositions was observed for the period investigated. By day 7, the release was highest in zinc free glasses (1064 ppm) followed by 10% mol zinc glass (950 ppm). A linear increase in ion release was also observed for cations over the 7-day period. Cation release increased with increasing dissolution rates, the least ion leaching was seen for the least soluble glass that is 3 mol % zinc phosphate glass. The release trend of sodium was like that of anion (PO_4^{3-}). There was no significant difference in the release rate of Na^+ between the compositions, containing 200-300 ppm.

On the other hand, calcium release was highest in glass without zinc oxide, followed by 1% zinc glass. This was expected as the glasses with highest CaO content were the most soluble. By day 7, between 100-300 ppm of calcium ions were leached from the given glass compositions, least calcium ion leached from 3 mol % zinc glass composition. As with Zn^{+2} ion release, an increase in the quantity of zinc ions was observed with increasing zinc oxide content in the glass compositions.

A



B



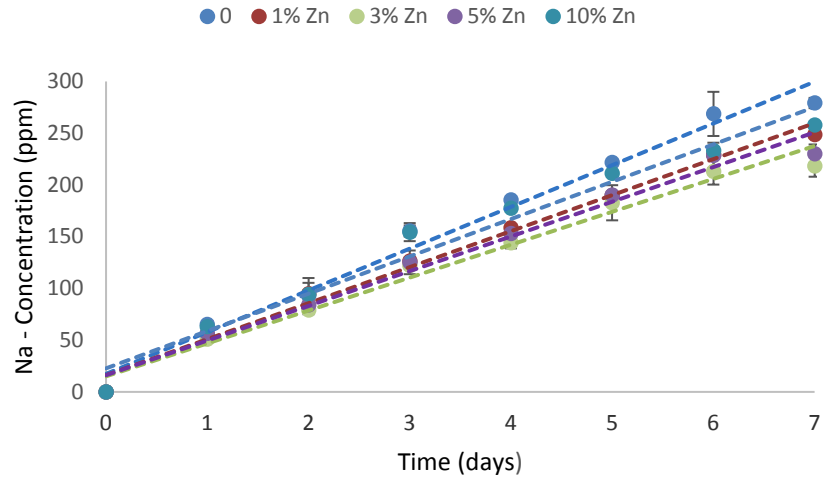
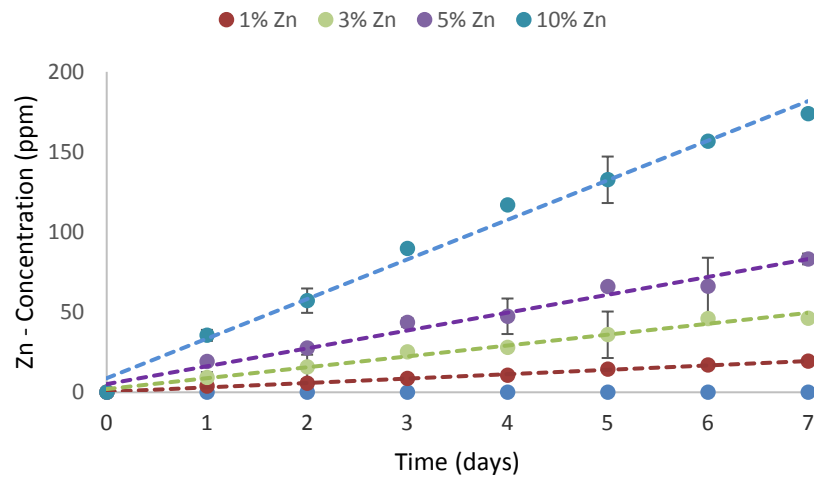
C**D**

Figure 2.8: Cumulative ion release (A) phosphorus, (B) calcium, (C) sodium and (D) zinc as a function of time for 0, 1, 3, 5 and 10 mol% cobalt doped glasses in distilled water. (Error bars = \pm SD for triplicate samples).

2.5 Discussion

Glass dissolution is a complex process which not only depends on the glass composition and structure but also on the other factors such as the surrounding environment i.e. ionic strength, temperature and the pH of the solution [11]. The aim of this study was to investigate the glass solubility with an increasing metal oxide content in different media, subsequent changes in the pH and ion release profile of the glasses.

The degradation rate of cobalt and zinc doped glasses showed variation in trend with increasing metal oxide concentration. Delahaye *et al.* [5] studied the dissolution kinetics of sodium-calcium metaphosphate glasses, according to their investigation the dissolution of the glasses is governed by the ionic strength of the solution; an increase in ionic strength of the surrounding environment decreases the weight loss with time. They postulated that the glasses with higher CaO content release more calcium ions which are likely to increase the ionic strength of the solution and subsequently decrease the dissolution rate of the glasses. In the present investigation it was observed that the glasses with higher calcium ions leaching had higher dissolution rates. Cobalt free glass composition had the highest release rate of cations and anions compared to cobalt containing glasses. The similar effect was also demonstrated by Franks *et al.*, [9] where a decrease in glass dissolution was observed with substitution of CaO with MgO.

A decline in dissolution of cobalt doped glasses was observed to occur with an increasing amount of cobalt oxide at the expense of calcium oxide. The observed trend in dissolution pattern fits well with the literature available, several studies have demonstrated that addition of metallic species decreased the dissolution of glasses with an increasing metal oxide content. A decrease in dissolution of silver doped phosphate glasses was observed by Ahmed *et al.* [12-15]. Brauer *et al.*, [16] investigated the effect of adding titanium oxide in phosphate glasses and showed that increasing the amount of titanium oxide decreased the solubility of glasses in water and simulated body fluid (SBF). Similar effect were seen by Moss *et al.*, [17] where addition of titanium

increased the chemical durability compared to titanium free composition $(\text{P}_2\text{O}_5)_{50} (\text{CaO})_{30} (\text{Na}_2\text{O})_{20}$. Ahmed *et al.*, reported systematic increase in the cross-link density, hence the chemical durability of MgO doped glasses compared to magnesium free glasses. Increase in gallium oxide content in phosphate glasses has also been shown to increase glass durability and hence decreased dissolution [18, 19].

The authors of the above-mentioned studies provided various explanations of the decreased dissolution behaviour with structural analysis of the glasses. However, it is difficult to make a direct comparison of the given work due to compositional variations. Even though no experimental study was undertaken to elucidate the change in glass dissolution, nonetheless the possibilities are explained here.

The dissolution of metaphosphate glasses is mainly controlled by the bonding of the phosphate chains to the modifier atoms [20]. As stated earlier in section 1.8, the basic structural unit of phosphate glasses is the orthophosphate tetrahedron surrounded by four oxygen atoms. Three of these oxygen atoms are available to form covalent bonds with phosphorus atom, P-O-P bonds. The addition of other components such as calcium or sodium breaks P-O-P bonds creating non-bridging oxygen atoms. The resulting phosphate chains form crosslinks between modifier cations through ionic bonds making the network more compact [21] and hence chemically durable. The strength of the ionic bond depends on the charge to size ratio of modifier cations. Since the ionic radius of cobalt is smaller than that of calcium, i.e. the charge to size ratio of cobalt is more than that of calcium therefore the bonds formed by cobalt are stronger than calcium. This makes the cleavage of the bonds difficult when cobalt is replaced with calcium making the glasses less soluble in aqueous media. Thus, when cobalt content is increased, the weaker Ca-O bonds are replaced by stronger Co-O bonds thus the dissolution decreases with an increasing cobalt oxide content.

Zinc doped glasses showed a similar trend in weight loss up to 3% compositions however, 5% and 10% mol zinc doped glasses showed a considerable increase in dissolution compared to 3% mol composition. The substitution of calcium oxide with zinc means high charge to size ratio that makes the crosslinked bonds stronger and therefore makes 1 and 3 % mol glasses more durable compared to zinc free compositions. However, the glass composition with higher zinc oxide content had higher degradation rates. The change in the dissolution behaviour could be explained based on the network clustering of modifier cations. According to Christie *et al.*, [20], clustering of modifier cations is more likely to occur at low concentrations which strengthens the network and vice versa. Therefore, it is postulated that higher zinc oxide content leads to open network therefore it is easy to ingress water and thus solubility increases. A similar trend in glass dissolution was observed by Salih and Mustafa [22, 23] where the highest zinc oxide containing glass showed significant weight loss compared to compositions containing lower zinc oxide content. A study conducted by Salih *et al.*, [22] on zinc glasses showed a higher dissolution rate of glasses with less calcium oxide and more zinc content. A detailed structural analysis of the glasses, beyond the scope of this thesis, is required to capture the structural changes in the glass network that affects the dissolution of zinc oxide glasses.

The dissolution rate of phosphate based glasses depends not only on their chemical stability but also on the nature of the media [3]. The difference in the dissolution rates in different media can be explained by the diverse composition of the media used. Nutrient broth contains sodium chloride whereas cell culture media contains various ions such as sodium, calcium, potassium, chloride and magnesium etc. along with amino acids, vitamins, growth factors and glucose. The components found in these formulations are also leached from glass. As the ionic strength of the solution is already higher than that of distilled water the dissolution of glasses is suppressed. Similar effect was observed by Uo *et al.*, [21] where the authors observed reduced dissolution rate of the glasses studied in SBF compared to distilled water. In media, such as nutrient broth and cell culture media which have neutral pH the system has sufficient capacity to neutralise the minimal changes occurring in surrounding environment due to glass leaching [2]. Since the pH

of the media also influences the rate of glass dissolution, the buffering capacity of nutrient broth and cell culture media suppress the glass dissolution. Bunker *et al.*, [2] investigated the effect of pH buffered media on glass dissolution and found strong dependence of glass dissolution on the pH of the solution. In strongly acidic or alkaline solution rate of dissolution increases dramatically. This was observed when glasses were dissolved in synthetic urine, an increase in dissolution by a factor of seven occurred. Gao *et al.*, [3] demonstrated a strong role of pH in the dissolution process as a dramatic increase in dissolution of the glasses was observed in extreme pH changes.

The ion release and pH results of both cobalt and zinc doped glasses are concurrent with the weight loss data. Increase in the release rate of anion and cations was observed in the most soluble compositions compared to less soluble glasses. With an increasing cobalt or zinc oxide content less calcium and more cobalt/zinc ions were leached. The pH analysis in most media showed the buffering capacity of the systems which also explains the suppressed dissolution.

2.6 Conclusion

A series of cobalt and zinc doped phosphate based glasses were successfully manufactured and characterised. The rate of dissolution of cobalt doped glasses was shown to decrease with an increasing cobalt oxide content whereas for zinc doped phosphate glasses, an increase in dissolution rate was seen with an increasing zinc oxide content. It was established that the glass solubility associated ion release is affected by several factors such as the glass structure, ionic strength and pH of media. A detailed structural analysis of the glasses is however required to capture the structural changes in the glass network that affects the dissolution of zinc doped glasses.

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Chapter 3

**Study of antimicrobial efficacy of cobalt or zinc
doped phosphate based glasses; alone.**

3.1 Introduction

The growth and attachment of microorganisms on surfaces leads to contamination and/or infections [1], therefore prevention of microbial adhesion and colonisation is the preeminent strategy to combat infections. In the last few decades many antimicrobial surfaces have been developed such as antibacterial plastics, ceramics and clothes [2-4] to prevent microbial colonisation. Likewise, recent attempts to coat or impregnate medical devices, such as urinary and central venous catheters, ventilators, dental and orthopaedic implants, with antimicrobials are gaining interest [5-7]. Microorganisms, like all living cells require trace elements such as Mn, Zn, Co, Mo, Ni and Cu for their growth and survival. However, a significant increase in the intracellular concentration of these metallic ions can be toxic. Heavy metals, therefore have long been used as antimicrobials. For example, in ancient times copper plates were used to sterilise drinking water and it has been recently used to coat door handles [8]. Silver nanoparticles have also been widely used in dental implants, catheters and burn wound dressings [9]. Thus, metal ions can act as antimicrobial agents and by using a controlled system can be delivered locally to prevent infections such as those caused by urinary catheterisation.

Bioactive glasses are of great importance not only due to their ability to establish chemical bonds with soft and hard tissue but also due to their broad antimicrobial properties [10, 11]. Given the importance of bioactive glasses, various glass compositions have been prepared to investigate their antimicrobial efficacy, a number of studies demonstrated antimicrobial effects of silica based bioactive glasses against various pathogenic microorganisms [11-15]. 45S5 has been extensively investigated for its bone tissue regeneration as well as its antimicrobial potential [12, 16-18]. Similarly, S53P4 bioactive glass has been shown to possess strong antimicrobial effect against four oral microorganisms [14]. The antimicrobial properties of these bioactive glasses have been attributed to the increase in the pH of the media that occurs due to leaching of ions, immersed in an aqueous environment [19-21]. However, an *in vivo* study conducted on the

antimicrobial effects of particulate 45S5 showed the lack of antimicrobial effect [13]. The human body, under normal physiological conditions, maintains a haemostatic state by keeping pH with in a specific range, thus the resulting pH change due to bioactive glasses diminishes due to body's buffering capacity and hence lack of antimicrobial activity. Following the discovery of 45S5 and S53P4, different glass compositions have been investigated to modify bioactive properties or to give them new properties by incorporating antimicrobial ions.

Silver and copper are the most widely studied metals for their broad and strong antimicrobial properties [4, 22-26], they have also been incorporated in bioactive glasses. Silver doped bioactive glasses have shown bacteriostatic and bactericidal properties against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Knowles *et al.* [24, 26] investigated the antimicrobial effect of silver-doped phosphate-based glass on planktonic bacteria as well as the effect of increasing copper content in phosphate based glasses on biofilms of *Streptococcus sanguinis*. Similarly, silver and gallium doped phosphate glasses have been shown to possess antimicrobial properties against Gram negative and Gram positive bacteria including MRSA [27-29]. Although silver and copper have been shown to exhibit antimicrobial properties, recent studies have documented emergence of resistance. McHugh *et al.* [30-35] reported the first silver resistant strain, *Salmonella typhimurium* and since then silver resistance has been described in members of the *Enterobacteriaceae*, *P. aeruginosa*, *Candida albicans*, *Escherichia coli*. Similarly copper resistant strains of *Staphylococci*, *Kocuria palustris*, *Brachybacterium conglomeratum*, *Sphingomonas panni* and *Pseudomonas oleovorans* have been reported [36]. Therefore, due to the emerging resistance it is essential to expand the range of antimicrobial glasses to combat existing infections and reduce the risk of developing resistant strains.

The antimicrobial properties of silver and copper have received much attention in the literature and other heavy metals have been overlooked. To determine the antimicrobial efficacy of metals,

such as cobalt, copper, gallium, nickel, gallium and zinc, a pilot study was conducted against a Gram positive and a Gram negative strain over a 4-day period (A.1 – A.4). The data demonstrated a strong antimicrobial effect of copper, cobalt and zinc within 24 hours compared to gallium, nickel and silver. Due to the promising results shown by cobalt and zinc metal powders their metal oxides were used to dope a ternary phosphate glass system (P_2O_5 - Na_2O - CaO) and the potential antimicrobial efficacy was evaluated. Since catheter associated urinary tract infections are caused by a diverse range of microorganism from Gram positive and negative bacterial species to fungi therefore, in the present study *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were used to study the antimicrobial efficacy of the manufactured glasses.

3.2 Aims

The aims of this study were to:

- Investigate the antimicrobial efficacy of cobalt and zinc doped phosphate based bio-glasses (1, 3, 5 and 10 mol %) against *Escherichia coli* (NCTC 10538), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (ATCC 76615).

This was achieved by studying:

- The effect on viability of microbial cells when in direct contact with cobalt or zinc doped phosphate based glasses using ISO-22196.
- Live/Dead staining of the microorganisms following a 24 hours exposure to glass surface.
- The effect of dissolution products (indirect contact) of cobalt or zinc doped phosphate based glasses using shaking flask method.

3.3 Materials and Methods

3.3.1 Microbial strains

Two bacterial strains; *Escherichia coli* (NCTC 10538) and *Staphylococcus aureus* (ATCC 6538) and a fungal strain, *Candida albicans* (ATCC 76615) were used in this study. These strains were maintained at -80°C stored on MicroBank beads (Pro-Lab Diagnostics Neston, Cheshire, UK). When required the beads were cultured in respective broth by incubating overnight at 37°C or 30°C (depending upon the strain) in an aerobic atmosphere. The inoculum was sub-cultured onto agar plates for short term storage at 5°C and used for experiments. The culture was adjusted in sterile broth to achieve the desired cell density for antimicrobial studies.

3.3.2 Microbiological media

Nutrient agar (NA), Nutrient broth (NB), Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were supplied by Oxoid Ltd (UK) and prepared by following manufacturer's instructions and autoclaved at 121°C for 15 minutes. The agar was allowed to cool down to 55°C and poured into sterile Petri dishes (Sarstedt Ltd, Leicester, UK). Plates and broths were stored at 4°C. Dey/Engley (D/E) neutralizing broth (Fisher Scientific, Leicester, UK) was also prepared as per manufacturer's instruction and stored at 4°C. Phosphate buffered saline (PBS) was purchased from Fisher Scientific (UK) and prepared by adding one tablet into 100 ml distilled water, this was then autoclaved for 15 minutes at 121°C to sterilize.

3.3.3 Standard curve for colony forming units using optical density

To determine the standard growth of the test microorganisms in the stationary phase, growth curves were plotted. Microorganisms were inoculated in respective broths (i.e. *E. coli* and *S.*

aureus in NB whereas *C. albicans* in SDB) and incubated overnight at 37°C and 30°C respectively. Following incubation serial dilutions were prepared in PBS in triplicates and the optical density (OD) of each dilution was determined at 570 nm using a spectrophotometer (Jenway 6305 UV/Visible Spectrophotometer). Cell viability was determined for each dilution using the spread plate method. The colony forming units of each dilution was then plotted against OD.

3.3.4 Antimicrobial activity of cobalt or zinc doped phosphate based glasses individually, against microorganisms in planktonic growth mode

3.3.4.1 Direct Contact – Surface testing

To determine the antimicrobial potential of the different glass surface, the international standard protocol for testing non-porous surfaces; ISO-22196 [37] was used. To conduct experiments an overnight microbial culture was adjusted using fresh Muller Hinton or Sabouraud dextrose broth depending upon the microbial strain. The adjusted cultures were inoculated on the top surface of the glass discs to produce $\sim 10^6$ CFU microbial density. A thin sterile glass cover slip (Fisher scientific, UK) was placed on the top of the inoculum and pressed gently so that the test inoculum spreads to the edges. The cover slip not only helps to create a thin bacterial film on the glass discs but also prevents the bacterial death due to desiccation. Standard plastic coverslips with 12 mm diameter were used as the control. Inoculated glass discs and control samples were then placed in a Petri dish and incubated in 37°C and 30°C incubators. At each time point; 1, 2, 4, 6, 24 and 48 hours two glass discs were removed from each set of glass composition along with untreated glass and washed with 5 ml of D/E broth (Becton Dickinson UK Ltd). This was achieved by placing the test glass discs with coverslips in 5 ml D/E broth and vortexed for two minutes. The viable bacterial count in the broth was determined by the spread plate method. In addition, immediately after inoculation, two untreated test specimens were processed and the viable count

determined was used as the recovery rate of the bacteria from the test specimens under investigation.

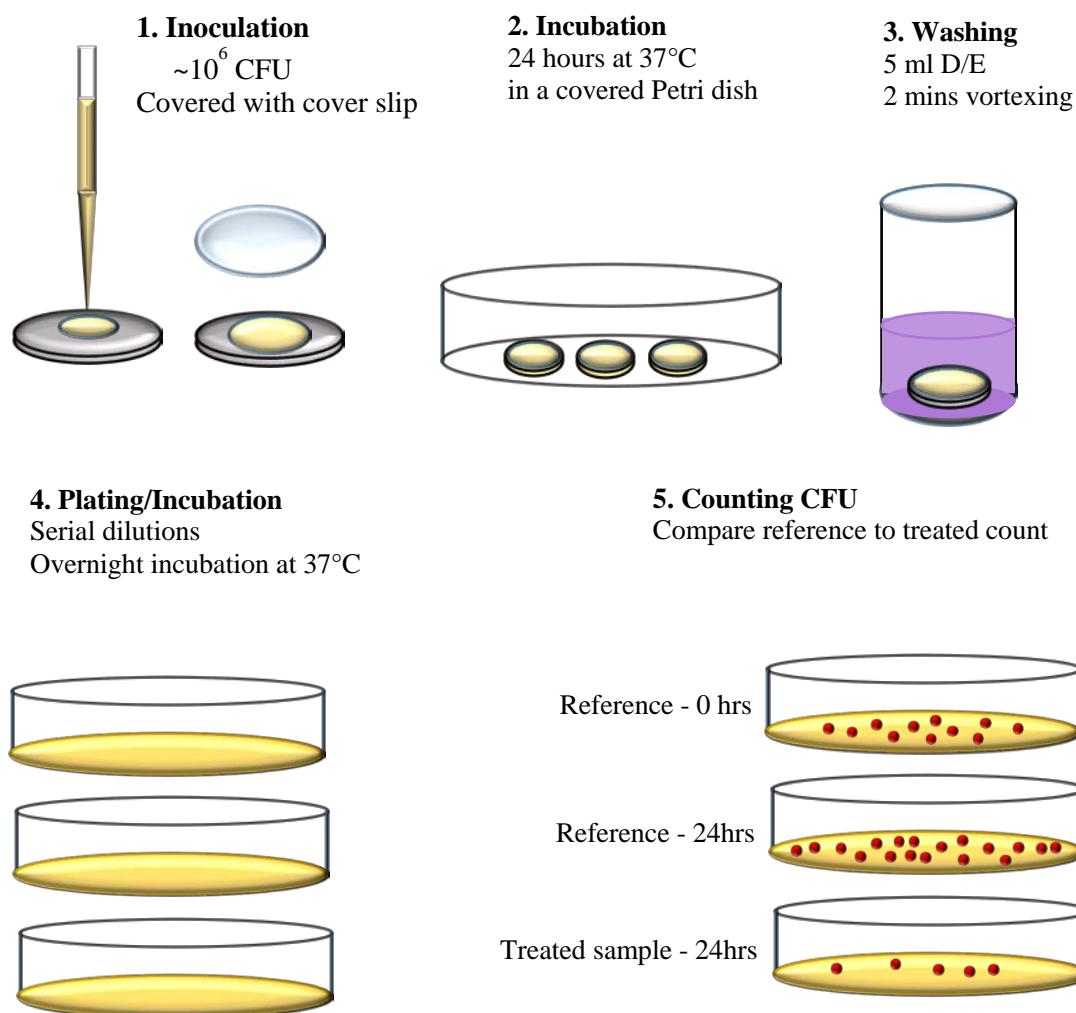


Figure 3.1: A schematic diagram showing the method for testing the antimicrobial activity of non-porous surfaces. The surface of sterilised test samples is inoculated with test microorganism and incubated for a period. Cell viability is determined to evaluate the antimicrobial efficacy of the material.

The antibacterial effect was evaluated according to the standard outlined in ISO-standard 22196.

The number of viable bacteria for each specimen was determined using equation 3.1:

$$N = (100 \times C \times D \times V)/A \quad \dots\dots\dots (3.1)$$

where N is the number of viable microorganisms recovered per cm² per test specimen;

C is the average plate count for the duplicate plates;

D is the dilution factor for the plates counted;

V is the volume in ml of D/E broth added to the specimen;

A is the surface area in mm² of the cover film.

The antibacterial activity, R was determined using the following equation

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \quad \dots\dots (3.2)$$

Where U₀ is the average of the common logarithm of the number of viable bacteria, in cells/cm² recovered from the untreated test specimens immediately after inoculation

U_t is the average of the common logarithm of the number of viable bacteria, in cells/cm² recovered from the untreated test specimens after time t respectively.

A_t is the average of the common logarithm of the number of viable bacteria, in cells/cm² recovered from the treated test specimens after time t.

3.3.4.2 Determination of Viable Non Culturable Cells by BacLight staining

Occasionally microorganisms under unfavourable conditions enter a state where they are metabolically active however cannot be cultured on media, because they do not reproduce and form colonies. Therefore, to confirm the findings of the direct contact experiment Live/Dead staining using fluorescent microscopy was undertaken to determine the viable but non culturable state of microorganisms. An overnight microbial inoculum was adjusted and inoculated on the top surface of the glass discs to produce $\sim 10^6$ CFU microbial density. A thin sterile glass cover slip was placed on the top of the inoculum and pressed gently so that the test inoculum spread to the edges. Following 24 hours incubation, fluorescent microscopy was conducted using the LIVE/DEAD® BacLight™ Bacterial Viability Kit purchased from Life Technologies (UK).

The LIVE/DEAD® BacLight™ staining kit uses two stains; SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. The stains differ in their ability to penetrate healthy cells, the SYTO® 9 penetrates all bacteria in a population, whereas propidium iodide penetrates only cells with damaged cell membranes. SYTO® 9 however when used with propidium iodide has reduced fluorescence when both dyes are present. Thus, live cells i.e. bacteria with intact cell membranes appear green whereas dead cells i.e. with damaged cell membranes stain red.

The stain was stored in the -20°C freezer and prior to use the stain was allowed to stand at room temperature for 30 minutes. Equal volumes (2 μl) of SYTO® 9 and propidium iodide were combined in a microfuge tube and mixed thoroughly by using a pipettor. 200 μl of sterile water was added to the microcentrifuge tube and mixed by vortexing for two minutes. The dye mixture was allowed to stand at room temperature for approximately 20 minutes. Following specimen's incubation, coverslips were removed and the glass discs were fixed onto glass slides using specimen mounting wax. The samples were then placed in sterile Petri dishes, 20 μl of the dye mixture was added onto each glass disc and incubated for 30 minutes at 37°C . Following

incubation unbound dye was removed with a paper towel and a glass coverslip was mounted onto the glass disc using mounting oil. The cells were observed under the x40 objective of the Leica DM16000 B microscope. All images were saved as TIFF files.

3.3.4.3 Indirect Contact – suspension analysis

Indirect contact analysis was used to determine the effect of dissolution products of glasses on the growth and viability of microorganisms tested. In this method, sterilised glass discs (Ø 10 mm, 2 mm thick) were placed in 25 ml of Mueller Hinton broth containing approximately 10^6 CFU/ml of the test microorganism. The samples were then incubated in an aerobic incubator at 30°C and 37°C with a shaking speed of 200 rpm. Mueller Hinton broth without glass discs served as negative control. At various time intervals; 24, 48, 72, 96 and 120 hours, a 100µl sample was taken out from each test container and serially diluted to determine the viable count using spread plate method. All experiments were undertaken in triplicate. A logarithmic microbial reduction of less than 0.5 and 1 was regarded as slight, values greater than 1 or less and equal to 3 as a significant and values greater than 3 as a strong antimicrobial activity [19, 38].

3.3.5 Statistical analysis

Two-way analysis of variance was carried out to determine statistical significances (GraphPad Prism 7.0). If a significant difference was detected a Tukey test was carried out to determine which values were significantly different. Differences were considered statistically significantly at a level of $P < 0.05$.

3.4 Results

3.4.1 Standard growth as determined by optical density

The standard curves for cell suspension of each microorganism using optical density produced linear graphs with R^2 values of 0.9203 for *E. coli*, 0.9604 for *S. aureus* and 0.9217 for *C. albicans* (Figures A5 - A7).

3.4.2 Antimicrobial activity of cobalt doped phosphate based glasses

3.4.2.1 Direct Contact – Surface testing

Figures 3.2 - 3.4 show the antimicrobial activity of cobalt doped phosphate glasses against *E. coli*, *S. aureus* and *C. albicans* at 2, 4, 6 and 24-hour time intervals. According to the ISO-22196, an antimicrobial activity greater than 2 demonstrates that the test specimen is antimicrobial.

Figure 3.2 shows the antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *E. coli* when cultured directly on glasses. Whilst minimum or no activity was seen in the initial hour (data not shown), all glass compositions demonstrated a strong antimicrobial activity within 2 hours when compared with non-treated control. At 2, 4 and 6 hours' time points, significant difference in R value was observed for 1, 3, 5 and 10 mol % cobalt doped phosphate glasses compared to un-doped phosphate glass samples ($p < 0.0001$).

Whilst a sharp increase in the activity was observed against *E. coli* within 2 hours, a gradual increase in antimicrobial activity was seen against *S. aureus*. As seen in figure 3.3, the R value remained below 2 for the initial 4 hours, but at 6 hours time point all glass compositions showed an antimicrobial activity ≥ 2 except for un-doped phosphate glass, however the difference observed was statistically non-significant. A similar trend in R value was observed at 24 hours.

The R value of all glass compositions exhibited a non-significant increase when compared to un-doped glass except for 10 mol % cobalt doped glass which showed a significant increase in R value when compared with un-doped phosphate glass ($p < 0.001$), 1 and 3 mol % cobalt doped glasses ($p = 0.0027$, $p = 0.0044$ respectively). Overall, whilst *E. coli* showed a higher susceptibility with a complete kill seen within 6 hours, complete killing of *S. aureus* was not seen within 24 hours.

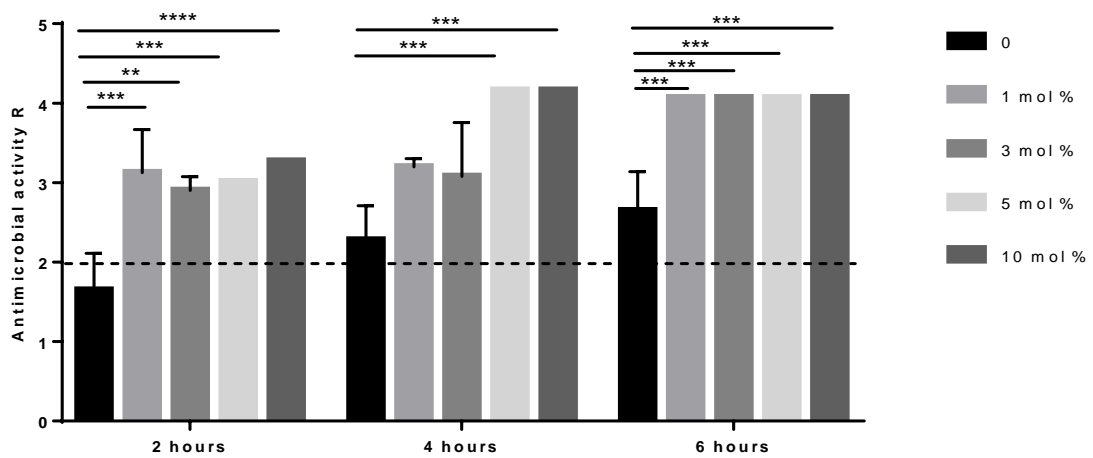


Figure 3.2: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *E. coli* at 2, 4 and 6 hours. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

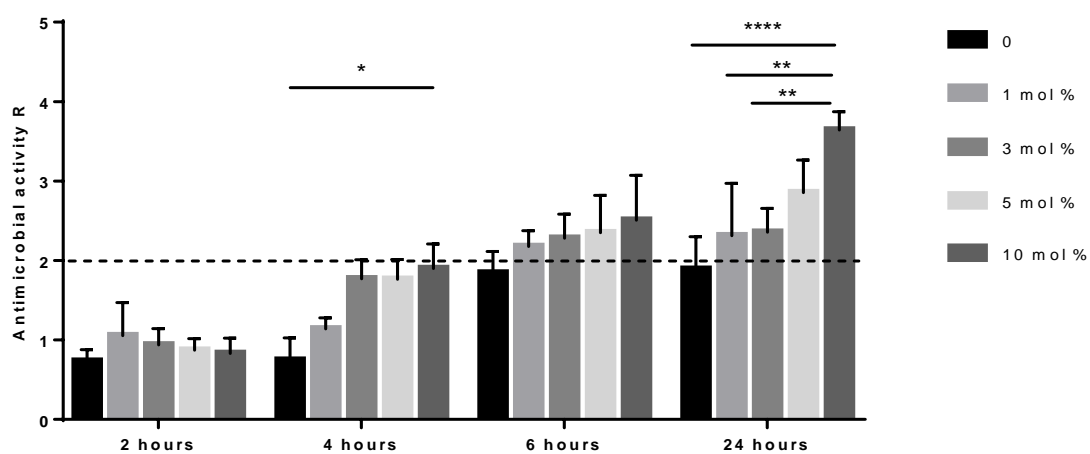


Figure 3.3: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *S. aureus* over a 24 hours period. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

Figure 3.4 shows the antimicrobial activity of the glasses against *C. albicans* when inoculated directly on glass discs. The R value in the first 2 hours remained below 2, however at 4 hours 3, 5 and 10 mol % cobalt doped glasses exhibited an antimicrobial activity ≥ 2 , whereas un-doped glass value was less than 2 highlighting the importance of incorporating antimicrobial ions. A statistically significant difference in the activity was not seen until 6 hours, 10 mol % cobalt doped glass showed a strong antimicrobial effect when compared to un-doped phosphate glass ($p = 0.0002$). A complete kill of *C. albicans* was seen within 24 hours.

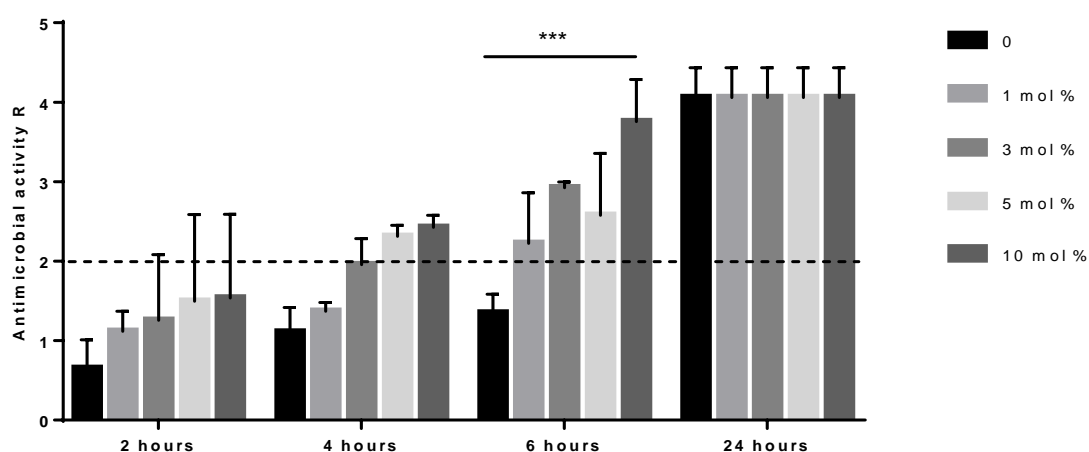


Figure 3.4: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *C. albicans* over a 24-hour period. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

3.4.2.2 Determination of Viable Non Culturable Cells by Baclight staining

Figure 3.5 – 3.7 shows the fluorescent images taken using Baclight stain following 24 hours incubation of *E. coli*, *S. aureus* and *C. albicans* respectively. The live cells take up SYTO[®] and appear green whereas dead cells appear red due to penetration of propidium iodide. It is demonstrated from the figures that the microorganisms are not viable following 24 hours exposure to glass surfaces and thus confirms the killing effect shown in direct contact analysis.

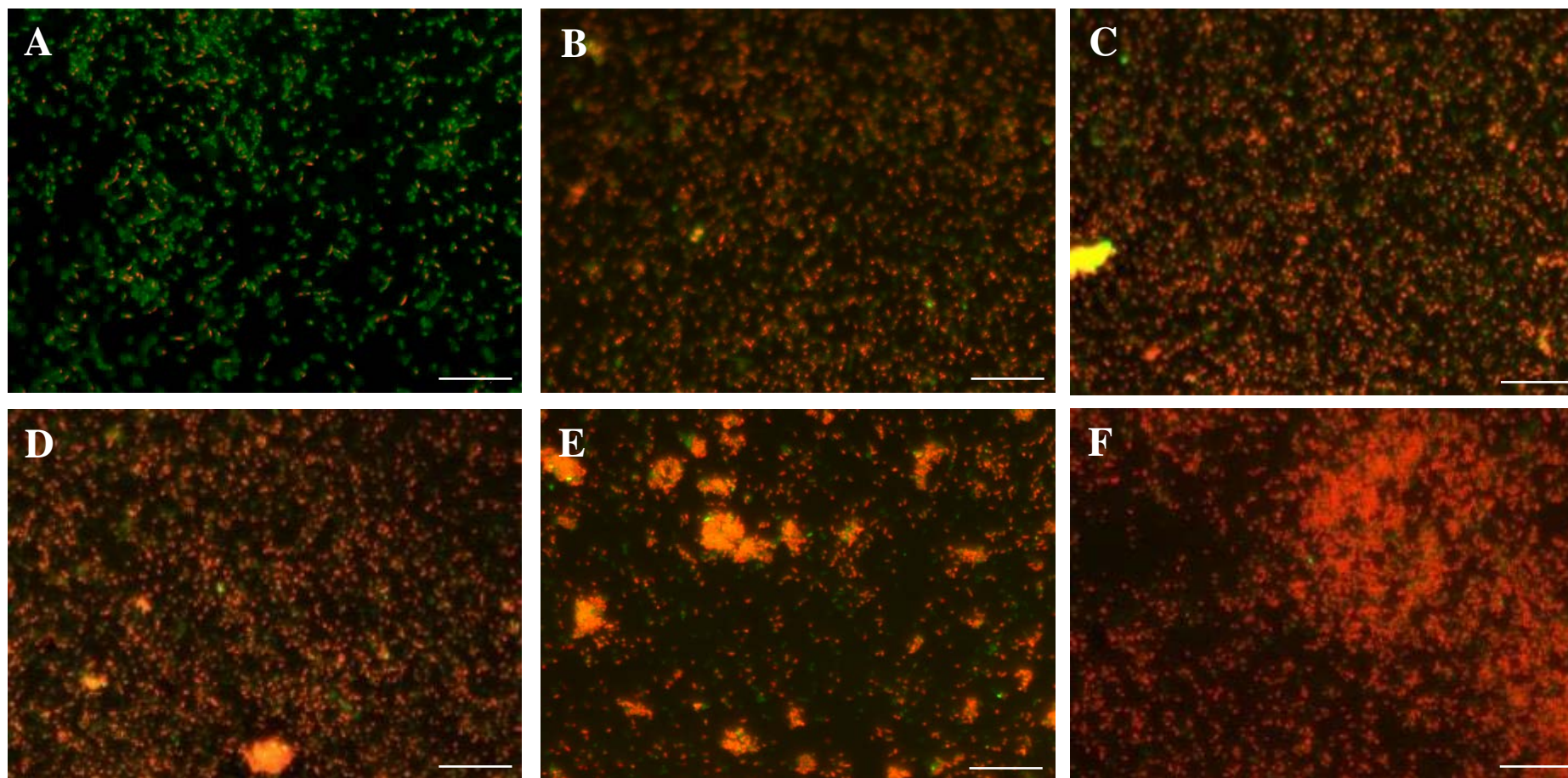


Figure 3.5: Fluorescent images taken by using Baclight staining of *E. coli* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % cobalt doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

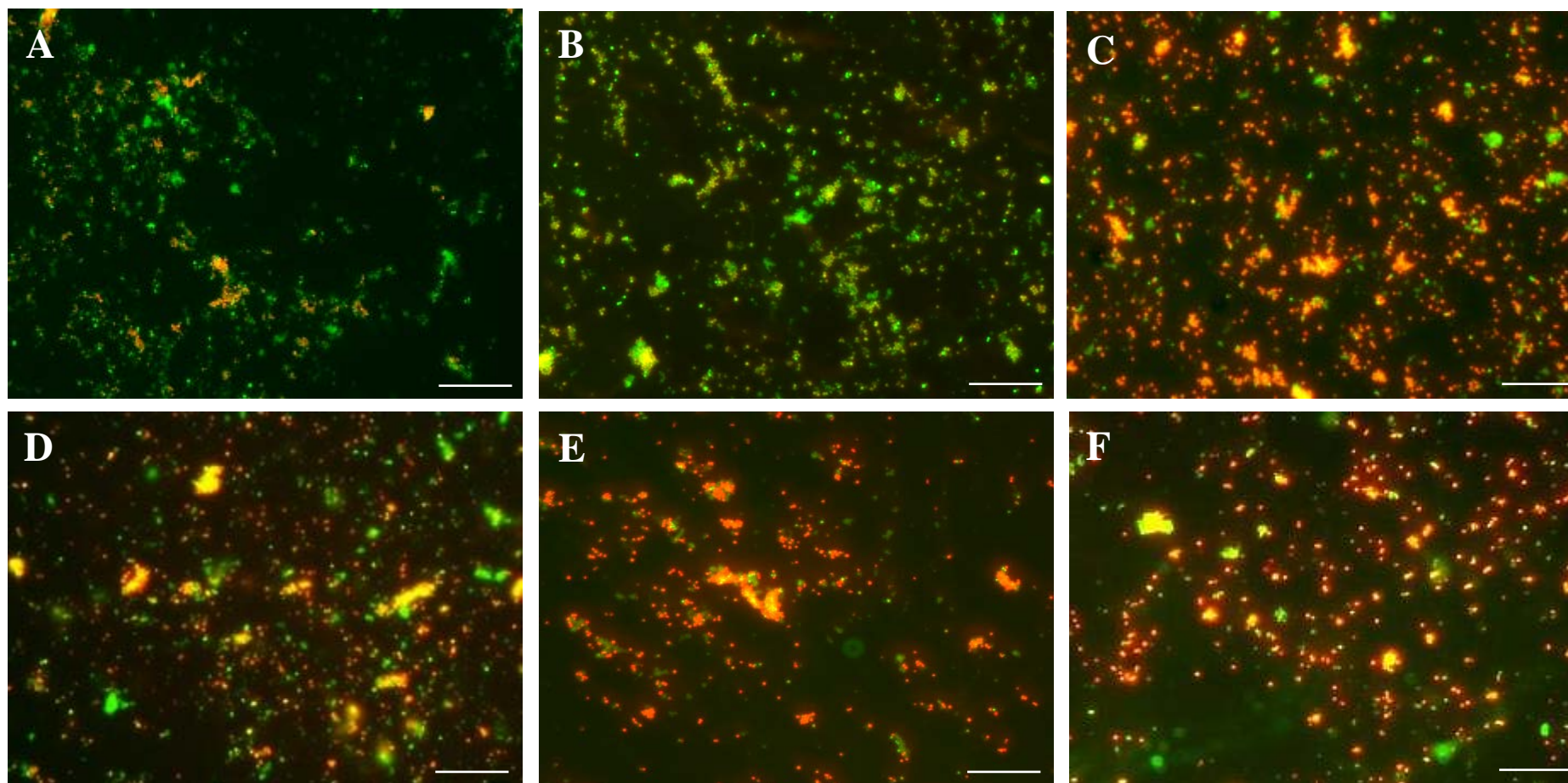


Figure 3.6: Fluorescent images taken by using BacLight staining of *S. aureus* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % cobalt doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

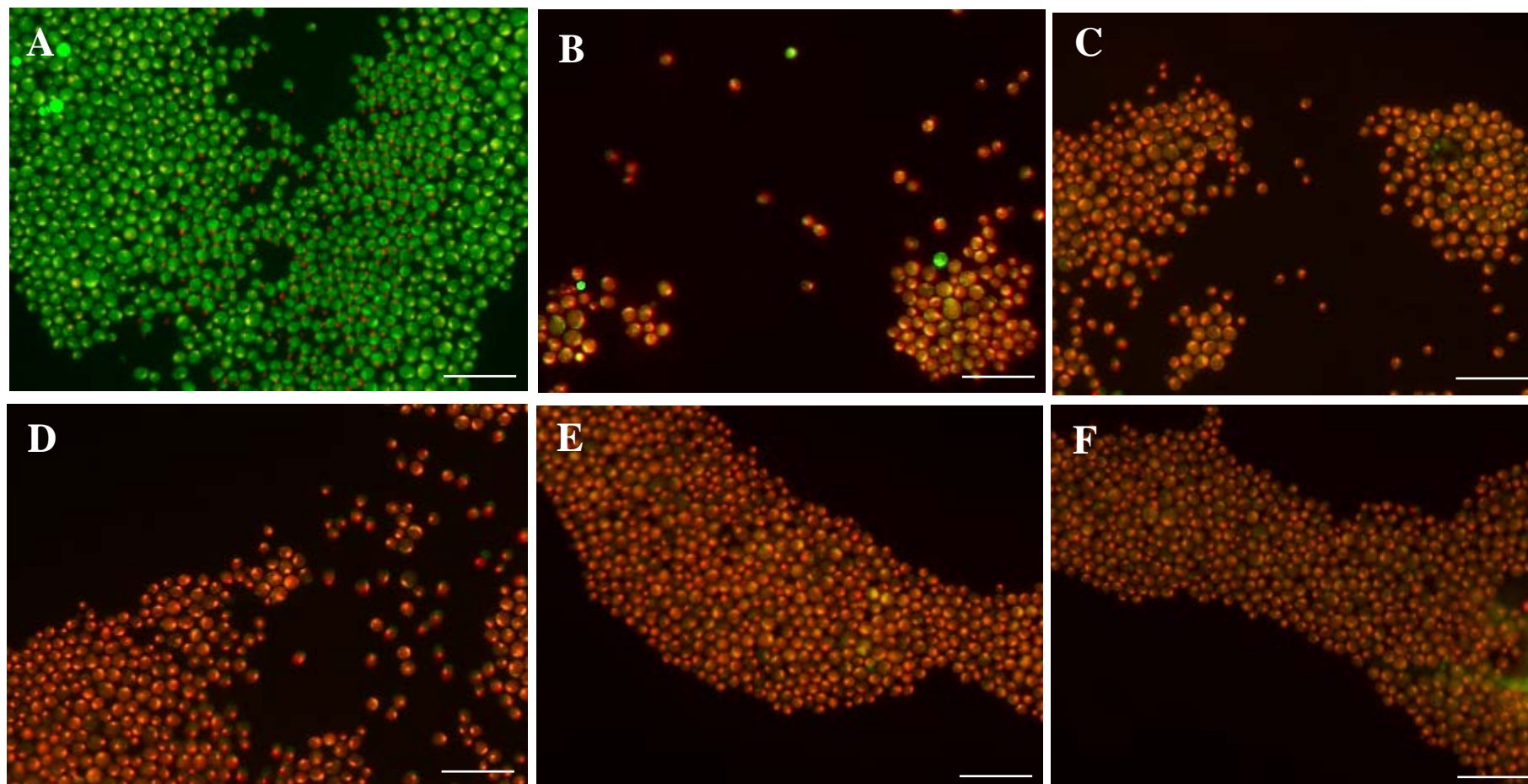


Figure 3.6: Fluorescent images taken by using Baclight staining of *C. albicans* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % cobalt doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

3.4.2.3 Indirect contact

Figures 3.8 – 3.10 show the effect of dissolution products of cobalt doped phosphate glasses over a 5-day period under an aerobic atmosphere at 37°C /200 rpm shaking speed. It was observed that the dissolution products of the glasses failed to demonstrate antibacterial effect against *E. coli* (Figure 3.8) over 5 days as the bacterial viability was approximately 100% when compared with microorganisms cultured in broth only. Similar results were seen against *S. aureus* (Figure 3.9) and *C. albicans* (Figure 3.10).

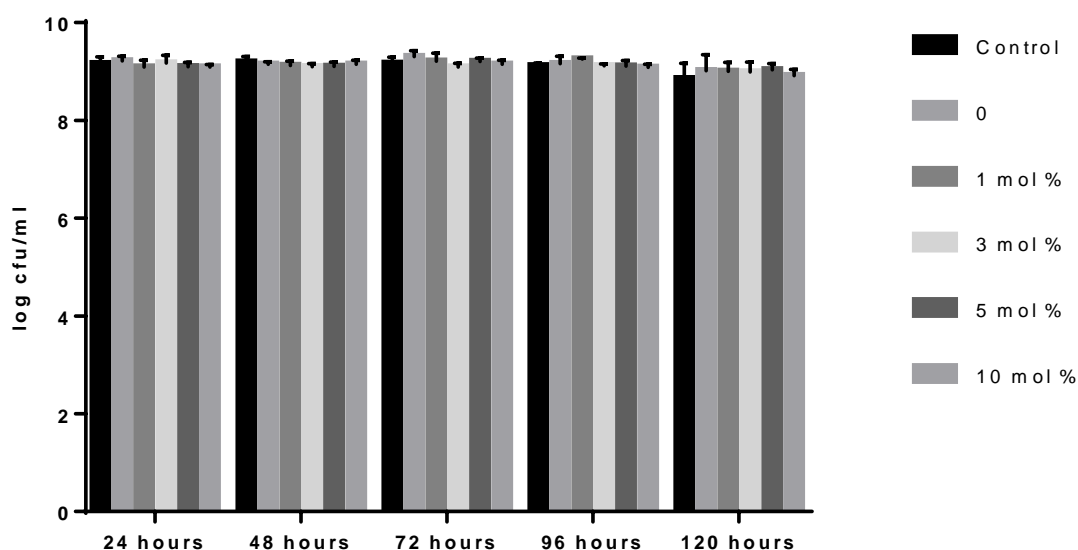


Figure 3.8: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *E. coli* over a 5 days period. Data shown are expressed as mean \pm SD (N=3).

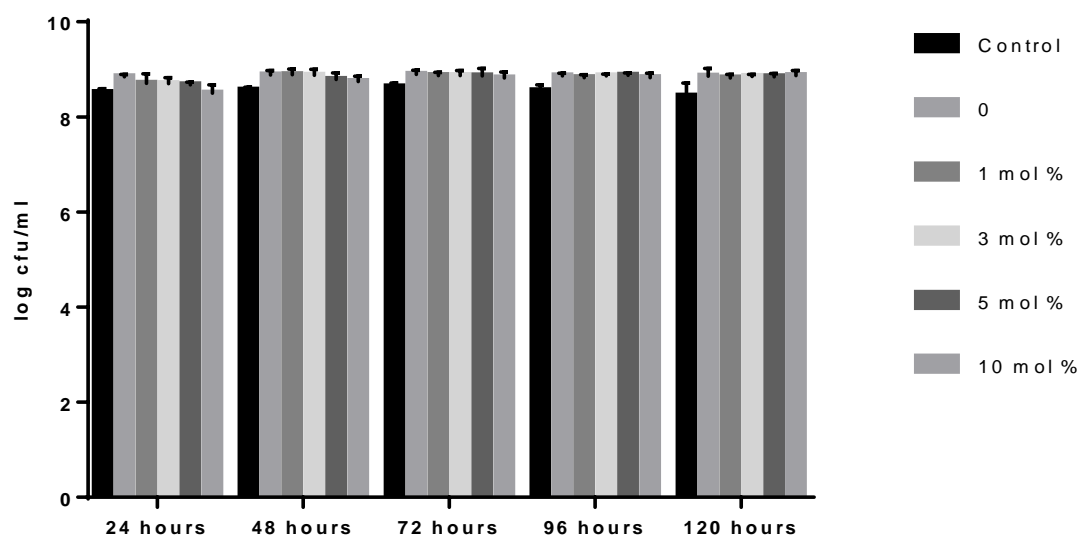


Figure 3.9: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *S. aureus* over a 5 days period. Data shown are expressed as mean \pm SD (N=3).

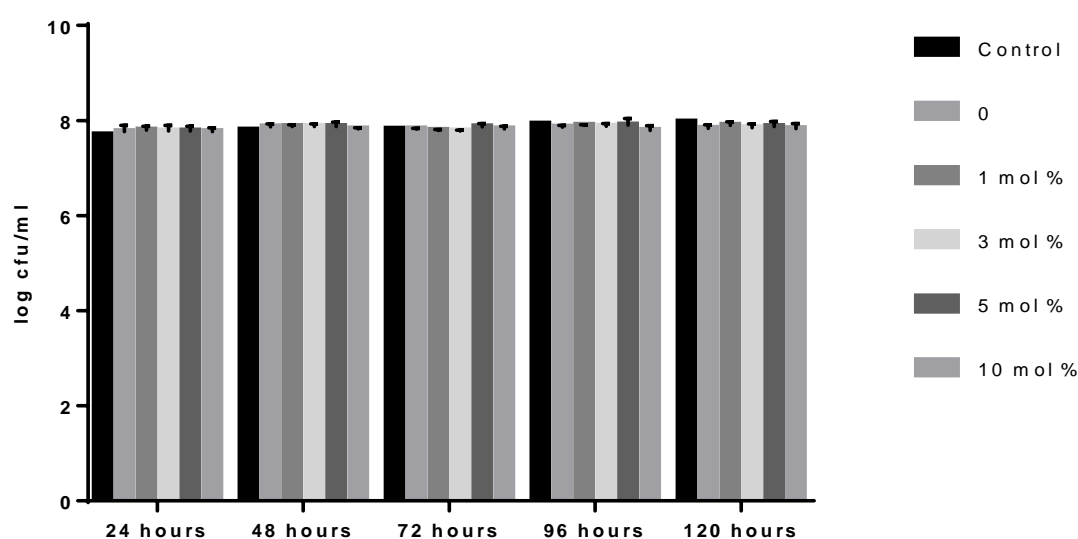


Figure 3.10: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % cobalt doped phosphate glass against *C. albicans* over a 5 days period. Data shown are expressed as mean \pm SD (N=3).

3.4.3 Antimicrobial activity of zinc doped phosphate based glasses

3.4.3.1 Direct Contact – Surface testing

The antimicrobial effect of 1, 3, 5 and 10 mol % zinc doped phosphate glasses compared to un-doped phosphate glass against *E. coli* is illustrated in figure 3.11. Zinc doped phosphate glasses showed an antimicrobial activity of ≥ 2 within initial 2 hours, however only 10 mol % zinc doped phosphate glass exhibited a significant difference when compared un-doped phosphate glass ($p = 0.0322$). Furthermore, at 4 hours, each zinc doped glass composition showed a significant difference in antimicrobial activity compared to un-doped phosphate glass ($p = 0.0233$).

Figure 3.12 shows the antimicrobial activity of zinc doped glasses against *S. aureus*. In the initial 2 hours, only 10 mol % zinc doped glass showed an antimicrobial activity of ≥ 2 , however the difference was non-significant when compared to un-doped phosphate glass. At 4 hours time point, zinc doped glass composition showed an antimicrobial activity greater than 2 and 10 mol % zinc doped glass exhibited a significant difference in comparison to un-doped phosphate glass ($p < 0.001$). A similar trend in antimicrobial activity was observed at 6 and 24 hours, however significant difference was only detected at 24 hours for 10 mol % zinc doped glass ($p = 0.0031$).

The antimicrobial activity of the un-doped and zinc doped phosphate glasses when tested against *C. albicans* can be seen in figure 3.13. It was found that at 4 hours 5 and 10 mol % zinc doped glasses demonstrated an antimicrobial activity above 2 which however was non-significant. A significant difference in the antimicrobial activity was not observed until 6 hours, 3, 5 and 10 mol % glasses showed a strong antimicrobial activity compared to un-doped phosphate glasses ($p = 0.0403, 0.0053, 0.0044$ respectively).

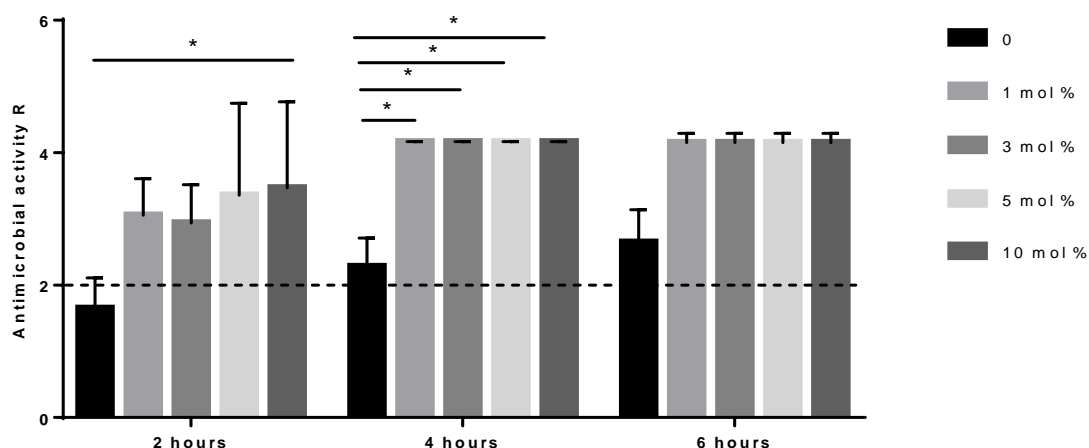


Figure 3.11: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *E. coli* over a 24 hours period. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

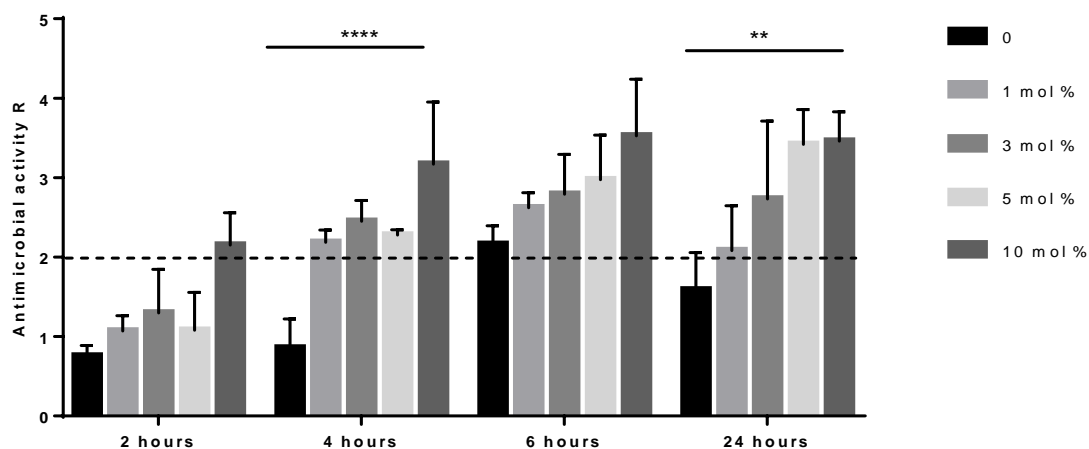


Figure 3.12: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *S. aureus* over a 24 hours period. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

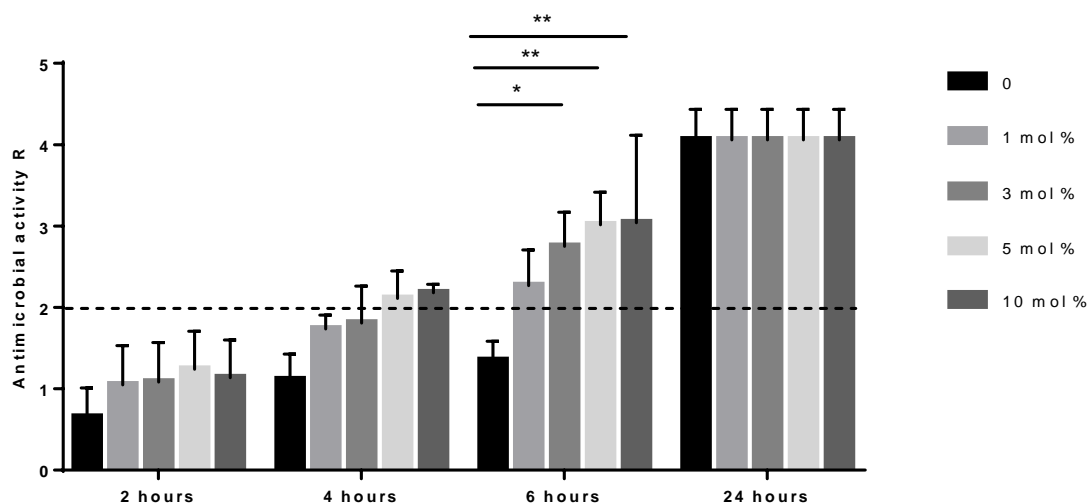


Figure 3.13: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *C. albicans* over a 24 hours period. Data shown are expressed as mean \pm SD (N=3) antibacterial activity as determined by ISO-22196.

3.4.3.2 Determination of Viable Non Culturable Cells by BacLight staining

Figure 3.14 – 3.16 shows the fluorescent images taken using BacLight stain following 24 hours incubation of *E. coli*, *S. aureus* and *C. albicans* respectively. The live cells take up SYTO[®] and appear green whereas dead cells appear red due to penetration of propidium iodide. It is demonstrated from the figures that the microorganisms are not viable following exposure to various compositions of zinc doped glass surfaces and thus confirms the killing effect shown in direct contact analysis.

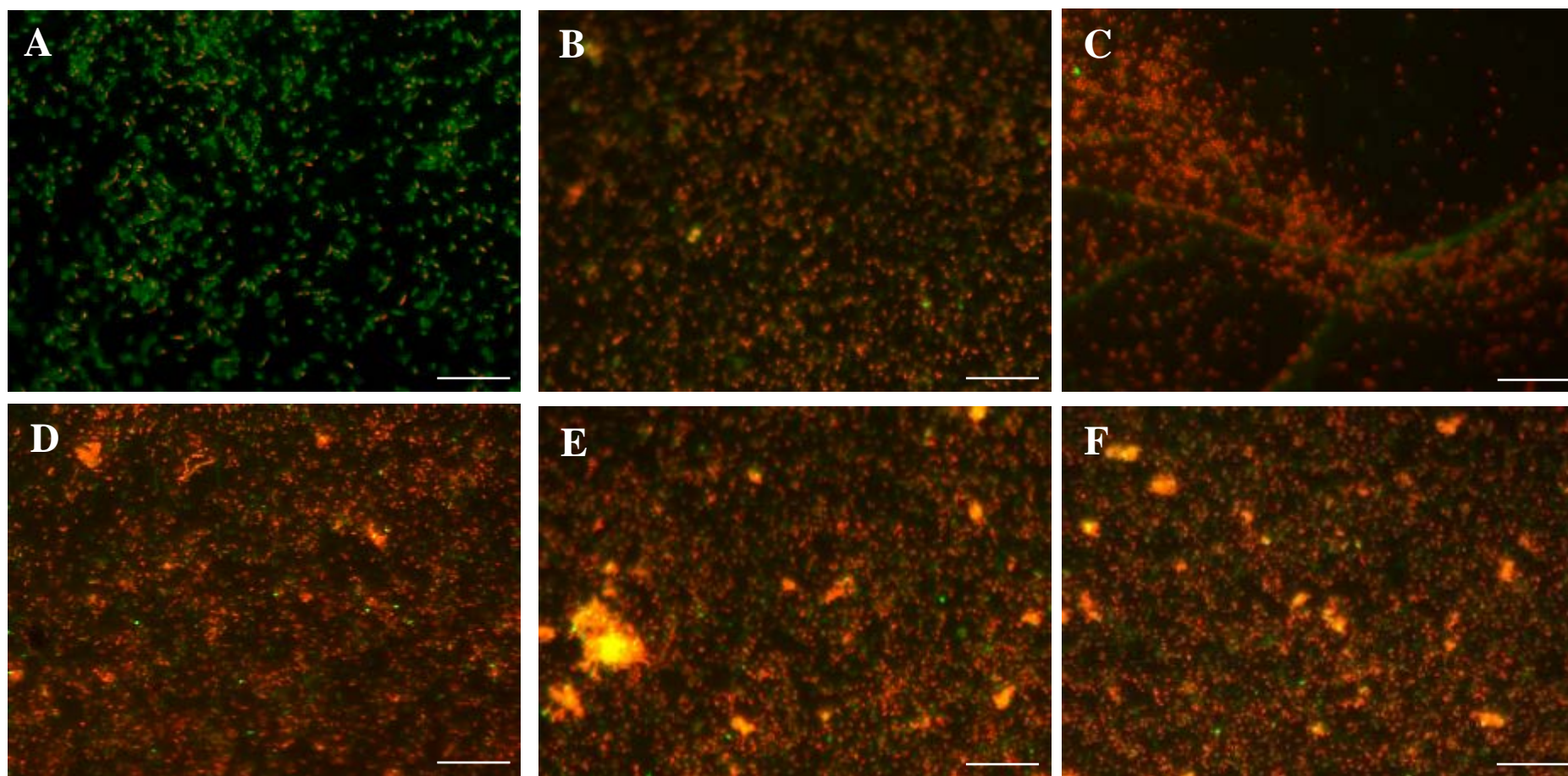


Figure 3.14: Fluorescent images taken using BacLight staining of *E. coli* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % zinc doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

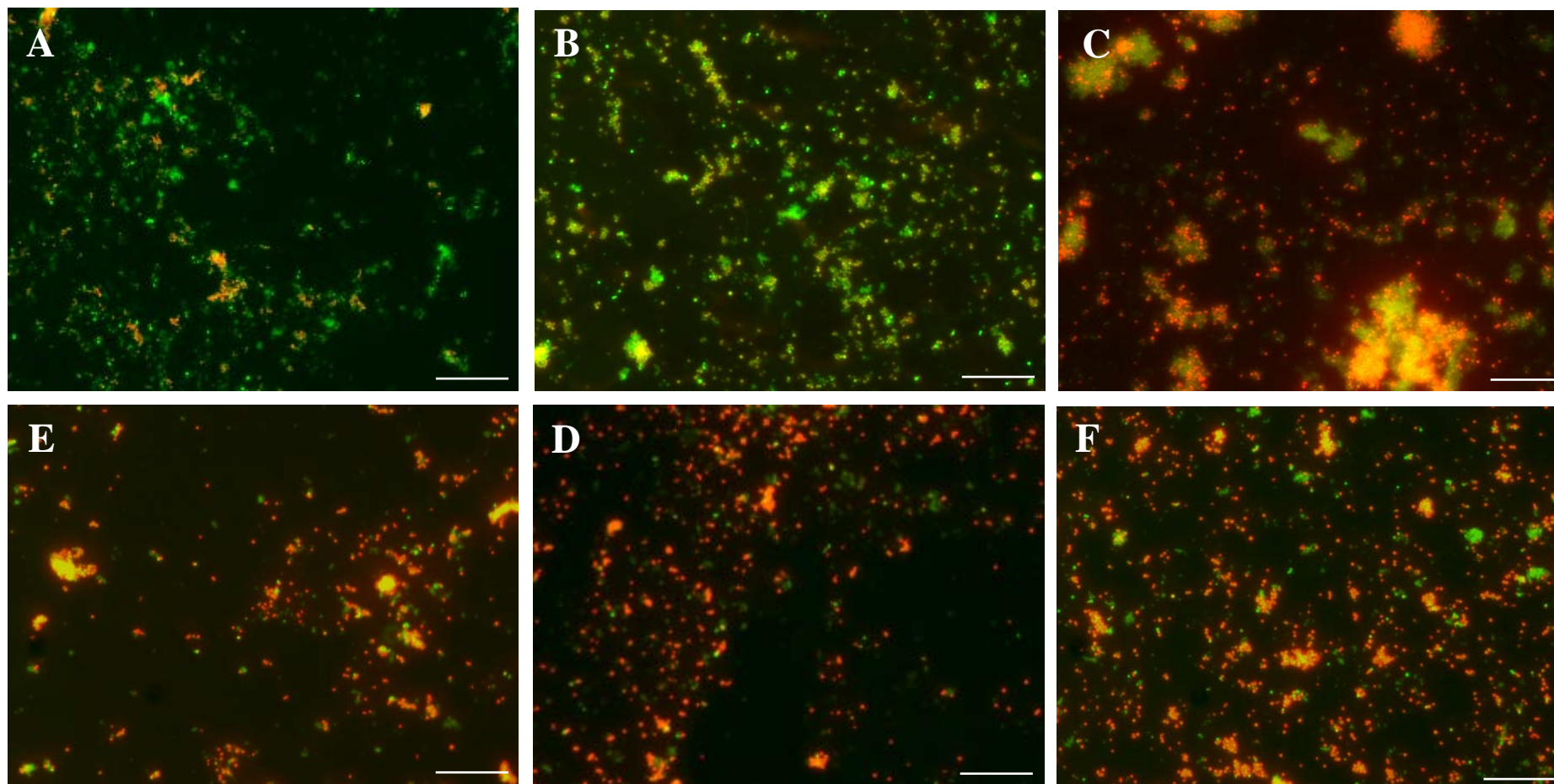


Figure 3.15: Fluorescent images taken using BacLight staining of *S. aureus* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % zinc doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

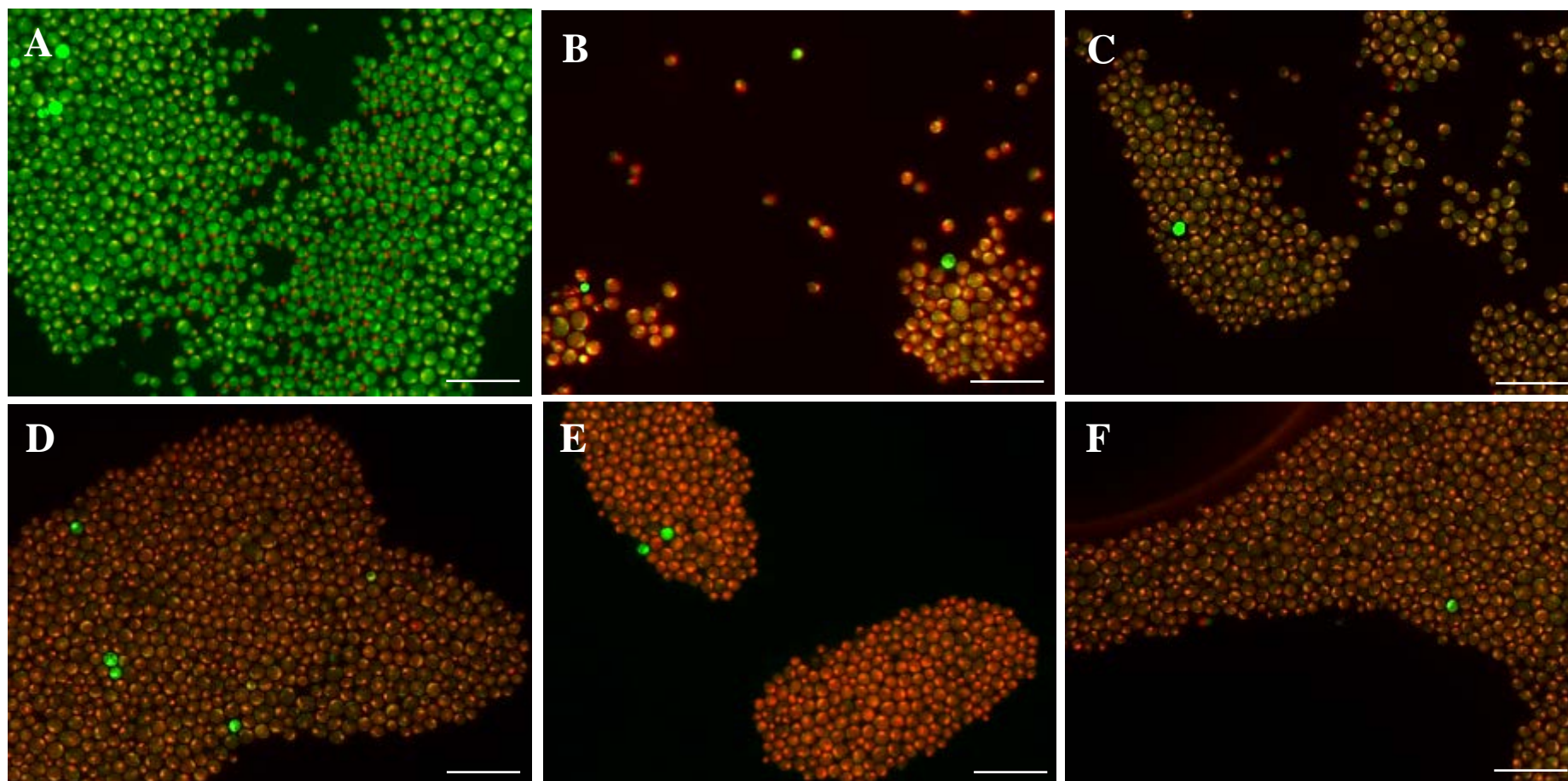


Figure 3.16: Fluorescent images taken using BacLight staining of *C. albicans* following 24 hours incubation on **A** - untreated control, **B** - un-doped phosphate based glass **C, D, E and F** 1, 3, 5 and 10 mol % zinc doped phosphate based glasses. Dead cells appear in red/yellow and live cells appear green. Scale bar represents 10 μm .

3.4.3.3 Indirect contact

Figure 3.17 shows the antimicrobial effect of the dissolution products of un-doped phosphate glass and zinc doped glasses against *E. coli* over 5 days period. It was found that dissolution products did not exhibit antimicrobial action for 96 hours. At time 120 hours the dissolution products of 5 and 10 mol % zinc glasses demonstrated a significant antibacterial effect when compared to control culture ($p = 0.0011$ and $p < 0.0001$ respectively). Additionally, when compared to un-doped phosphate glass, the antibacterial effect of 5 and 10 mol % zinc doped glass was significant ($p < 0.0001$). Significant differences in the antimicrobial activity was also seen for 5 and 10 mol % glasses compared to 1 and 3 mol % zinc doped glasses (Figure 3.17).

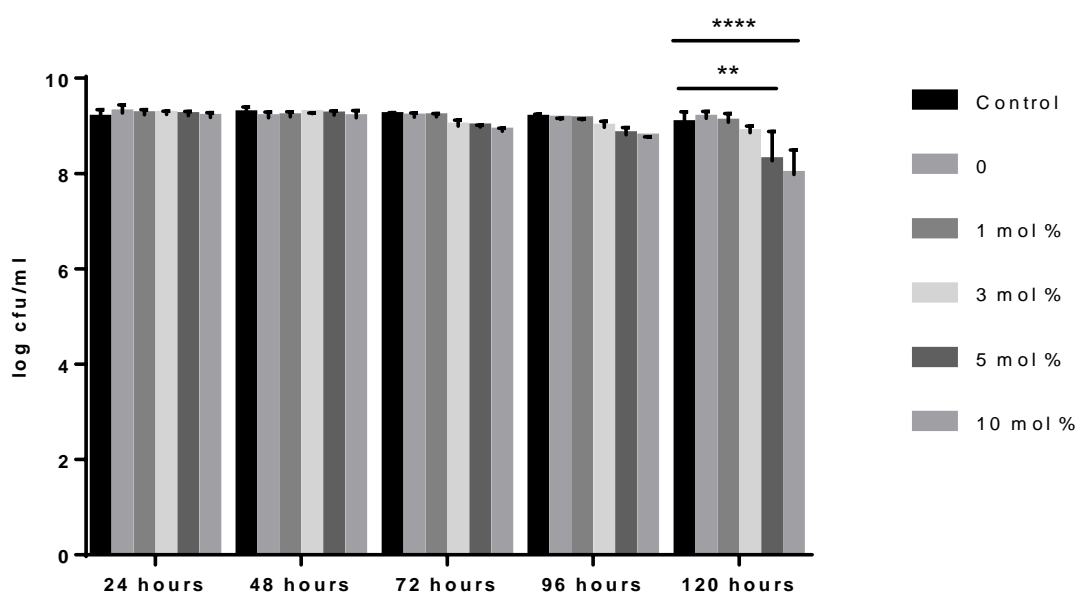


Figure 3.17: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *E. coli* over a 5 day period. Data shown are expressed as mean \pm SD (N=3).

The antimicrobial effect of the dissolution product of the glasses when tested against *S. aureus* is shown in figure 3.18. During the initial 24 hours, the dissolution products of the glasses failed to show antibacterial effect, similarly all glass compositions exhibited a non-significant decrease in bacterial log reduction at 48 hours except for 10 mol % zinc doped glass ($p < 0.0001$). Both 5 and 10 mol % zinc doped glasses exhibited a significant decrease in bacterial viability at 72, 96 and 120 hours compared to control culture as well as un-doped phosphate glass dissolution products ($p < 0.0001$). Similarly, the dissolution products of 5 and 10 mol % zinc doped glasses exhibited a significant decrease in bacterial density compared to 1 and 3 mol % dissolution products of zinc doped glasses. Interestingly, dissolution products of un-doped phosphate glass seemed to improve bacterial viability compared to control *S. aureus* cultures. A significant difference ($p = 0.0067$) was detected at 24 and 48 hours.

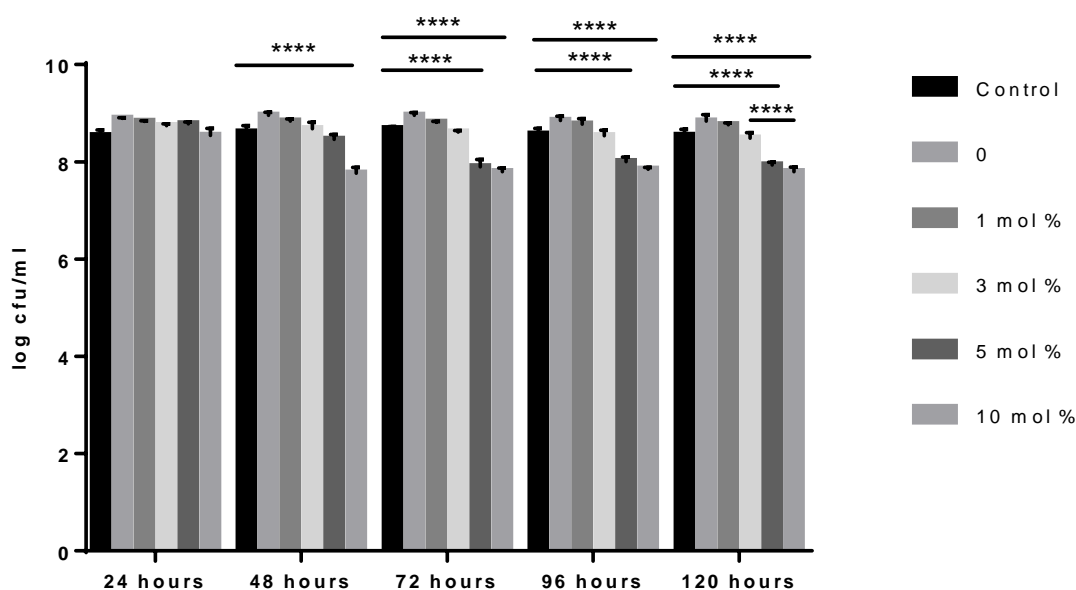


Figure 3.18: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *S. aureus* over a 5 days period. Data shown are expressed as mean \pm SD (N=3).

The dissolution products of zinc doped glasses, unlike against *E. coli* and *S. aureus* showed a strong antimicrobial activity against *C. albicans* in the initial 24 hours. The dissolution products of both 5 and 10 mol % glasses exhibited a significant decrease in microbial load compared to control, un-doped and 1 mol % glass dissolution products within 24 hours ($p < 0.001$). Furthermore, 3 mol % zinc doped dissolution products also showed antimicrobial effect against *C. albicans* ($p = 0.0133$). However, the antimicrobial effect seemed to diminish after 24 hours as the viability of *C. albicans* seemed to improve over the next two days and reached to the same density as the control cultures.

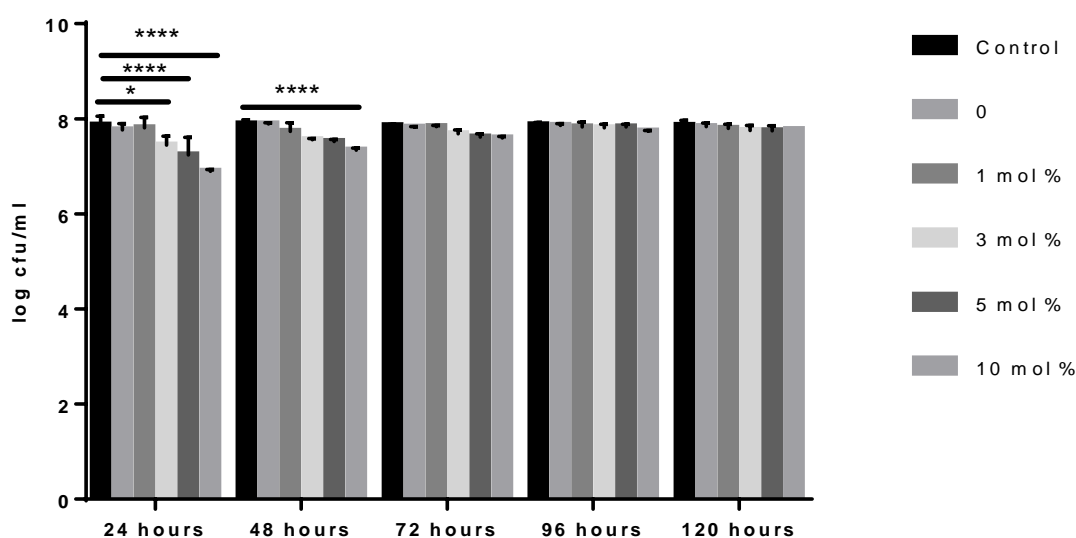


Figure 3.19: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *C. albicans* over a 5 days period. Data shown are expressed as mean \pm SD (N=3).

3.5 Discussion

As stated in section 3.1, many studies have been undertaken to elucidate the antimicrobial potential of various silicate and phosphate glass systems. However, to date, none of the studies have evaluated the antimicrobial effect of cobalt and zinc doped glasses in the context of controlled ion release for treating catheter associated urinary tract infections. The present study, therefore investigated the antimicrobial effect of an un-doped and doped (with 1, 3, 5 and 10 mol % cobalt or zinc oxide) phosphate based bioactive glass (P_2O_5 - Na_2O - CaO) against clinically relevant microorganisms.

The antimicrobial effect was determined using direct and indirect contact (dissolution products) method. The results of direct contact showed a strong antimicrobial activity exhibited by cobalt and zinc doped glasses compared to control specimens against *E. coli*, *S. aureus* and *C. albicans*. In addition, the effect was time dependent as a gradual decrease in microbial viability was observed over a period. Whilst an antimicrobial effect was seen against the three strains studied, *E. coli* showed higher susceptibility compared to *S. aureus* and *C. albicans*. Similar results were reported by Hu *et al.*, [16] demonstrating a high kill against *E. coli* when treated with Bioglass® compared to *S. aureus*. This is also in agreement with a study conducted on antimicrobial effect of gallium doped phosphate glasses where the authors observed a larger zone of inhibition for *E. coli* and *P. aeruginosa* when compared to *S. aureus* [29]. Likewise, *E. coli* was shown to have a higher susceptibility compared to *S. aureus* when treated with 3 mol % silicate based gallium glass [19].

The difference in the activity could be explained based on the differences in the outer cell wall of the Gram positive and Gram negative bacteria. Gram positive bacteria such as *S. aureus* contain a thick peptidoglycan layer in its cell wall along with teichoic and lipoteichoic acids. Teichoic acids are polyol phosphate polymers with a strong negative charge whereas in Gram negative

bacteria these are not present [39, 40]. These charged anionic clusters contribute to the binding of ions such as sodium, calcium, copper and cobalt etc. thus promote bacterial adhesion to the metallic surfaces. This cationic sequestering mechanism therefore confers resistance to bacterial killing in Gram positive bacteria. Furthermore, thick peptidoglycan layer protects bacteria from penetration of ions and reactive oxygen species generated by metallic ions [41], therefore confers stability to Gram positive cells. A thick peptidoglycan layer is believed to prolong the killing process as demonstrated by Espirito Santo *et al.*, [42, 43] where it was shown that *Staphylococcus*, when exposed to metallic copper surface, takes seven times longer to kill compared to *E. coli*. On the other hand, Gram negative bacteria contain an outer membrane containing lipopolysaccharides (LPS) which acts as a barrier for transport of antimicrobials. The passage through the outer membrane is regulated through non-specific ion channels called porins. These channels are hydrophilic thus do not allow the passage of hydrophobic molecules such as antibiotics, however metal ions can easily pass through porins and thus bring about the cell death [44-46].

Although the exact mechanism of action is not known, yet several factors have been shown to play a role in contact mediated killing by metallic surfaces. Direct contact of microbes with an antimicrobial surface damages the outer membrane by punching holes, such as shown by copper ions [8] which leads to loss of membrane potential. Additionally, reactive oxygen species are produced at the interface of bacterial cell wall and the bioactive glass surface causing further structural damage to the membrane. The loss of membrane integrity results in leakage of the cell contents and thus cell death occurs [47]. Similar mechanism was proposed in a study conducted on metallic copper surface against *S. aureus*. The authors showed a reduced level of intracellular copper ions following a complete kill which suggested membrane leakage [43].

The indirect contact experiments were conducted to investigate the antimicrobial efficacy of metal ions leached from the given glasses. The results of the present study show that the optimum release of cobalt ions required to illicit a toxic effect was not achieved in the test media, as the dissolution products failed to exhibit bacteriostatic/bactericidal effect against any of the test strains. The ionic concentration of 1, 3, 5 and 10 mol % cobalt approximately reached up to 5, 10, 14 and 17 ppm respectively. Whilst 1, 3, 5 and 10 mol % cobalt glasses failed to exhibit any antimicrobial effect, 5 and 10 mol % zinc doped glasses (32 and 66 ppm respectively) produced a considerable kill at 120 hours against *E. coli*. Likewise, significant difference in the activity was seen against *S. aureus* within 48 hours which improved by 120 hours. *C. albicans* on the other hand, showed antimicrobial effect within 24 hours which appeared to diminish as the time progressed. The dissolution products therefore showed minimum or no activity compared to direct contact experiments.

In indirect contact assays, the efficacy of bioactive glass depends on their solubility and release of constituent ions such as calcium, sodium, phosphorus, cobalt or zinc. These ions then enter the microbial cells via non-specific ion channels and can target various cell components directly and indirectly to bring about cell death. Metal ions produce reactive oxygen species that results in oxidative damage to DNA, mitochondria and proteins restricting cell growth and metabolism [48, 49]. The ions can also affect bacteria indirectly by binding to enzymes and inducing conformational changes thus making them non-functional. Subsequently bacteria fail to synthesize their machinery to carry out metabolic processes and hence death occurs. Cobalt ions have been shown to produce hydroxyl radicals whereas zinc ions result in production of superoxide molecules that subsequently target various cell components. Zinc ions are also believed to inhibit glutathione reductase, an enzyme that prevents oxidative damage in cells. Several bacterial species require manganese for carrying out metabolic functions, however zinc ions have been shown to competitively bind to manganese receptors and inhibits its uptake and thus exerts toxic effect on bacteria [50, 51].

Since the presence of metal ions is essential therefore the lack of antimicrobial activity could be due to reduction in the availability of free ions in solution. Microbiological media such as nutrient broth provides a complex environment with a number of ionic species that may reduce the concentration of free metal ions by precipitation or by the formation of soluble complexes [52]. Silver ions have been shown to form insoluble silver salts when in contact with large amounts of ions such as chlorides and amino acids present in broth, thus causing inactivation of silver ions [53]. Similarly, a study on zinc doped bioactive glasses showed that the amount of zinc ions leached from the glass was reduced by the formation of complexes between zinc ions and components of broth [54]. Contrary if the dissolution products are available as free ions, then the poor antimicrobial activity could be due to the neutralization mechanism deployed by the microorganism. Pathogenic microbes have evolved mechanisms to efflux heavy metals such as cobalt and zinc. *E. coli* for instance has efficient periplasmic efflux pumps that detoxify the bacterial cell in the presence of excess silver and copper [55, 56]. Therefore, it is reasonable to suggest that microorganisms have evolved similar mechanisms, such as efflux pumps, to remove excess cobalt and zinc ions.

The lack of activity in the suspension experiments could also be due to the buffering capacity of nutrient broth as demonstrated in the pH analysis. One of the most widely accepted mode of action of glasses in solution is via a rapid change in the pH of the surrounding medium. A study by Brown *et al.*, [57] confirmed that a rapid change in the pH causes reduction in bacterial viability. Similar results were also reported by Stoor *et al.* and Allan *et al.* [12, 14] therefore we can argue that the lack of activity of the glasses observed in the indirect contact study is likely due to the neutralization of the pH to near optimal range, as shown previously.

3.6 Conclusion

An antimicrobial activity was demonstrated by all glass compositions particularly by 5 and 10 mol % cobalt and zinc doped glasses when in direct contact with microorganisms. The killing effect was not only time dependent but also strain specific. The significant difference in the antimicrobial activity of un-doped and doped phosphate glasses suggests the additional benefit of adding cobalt and zinc ions as antimicrobials. The lack of activity in shaking flask experiment is either because the number of antimicrobial ions released was well below the concentration needed for killing microbes or due to inactivation of antimicrobial ions. The pH buffering and inactivation of metal ions raises concerns over use of these glasses *in vivo*.

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Chapter 4

**Study of antimicrobial efficacy of 5 mol %
copper, cobalt and zinc doped phosphate based
glasses; alone and in combination**

4.1 Introduction

Everyday many patients are prescribed antibiotics to treat bacterial infections which in some cases can prove effective, but inappropriate and overuse of broad spectrum antibiotics has led to a drastic increase in antibiotic resistance. The emergence of resistant strains and decline in discovery of new antibiotics has led to the idea of combining various antimicrobials to treat resistant strains and/or polymicrobial infections. Two or more antibiotics can work simultaneously to give additive, synergistic, antagonistic effect or without any effect on each other [1]. The interaction between any two antibiotics is considered synergistic if the combined effect is stronger than an additive expectation and antagonistic if it is weaker [2].

The idea of combining antibiotics to enhance their effect is not new; clinical interest started in the 1950s when streptomycin was added with penicillin G to treat enterococcal endocarditis [3]. Since then several antimicrobials have been combined and resulted in synergism [4], demonstrating beneficial effect in clinical medicine. Antibiotic combinations that inhibit cell wall and protein synthesis, beta-lactamase inhibitors with beta-lactam antibiotics and combination agents that inhibit sequential metabolic or synthetic pathways have been shown to exhibit synergistic effects [5]. Extensive *in vitro* data is available on antibiotic synergism, for instance, aztreonam is often used with clindamycin or another beta-lactam antibiotic [4]. Similarly, amdinocillin has demonstrated synergism when combined with other penicillins and cephalosporins [5]. A study conducted by Stephanie *et al.* [6] compared the effects of different combinations of a beta-lactam, vancomycin, and/or an aminoglycoside against 32 clinical strains of MRSA and demonstrated synergistic effects of vancomycin combined with imipenem or cefazolin, and even with netilmicin in a triple combination.

The antimicrobial combinations are not only limited to antibiotics but many other compounds with antimicrobial potential have been combined with antibiotics to enhance their activity. For

instance, antimicrobial peptides have potent antimicrobial action and have been combined with conventional antibiotics. Zhou and Peng investigated the interaction between polycationic peptides and clinically used antimicrobial agents in the treatment of clinical isolates of Gram-positive and Gram-negative aerobic bacteria [7]. A synergistic effect was shown between ranalexin and polymyxin E, doxycycline and clarithromycin. Similarly, magainin II was demonstrated to be synergistic with ceftriaxone, amoxicillin clavulanate, ceftazidime, meropenem, piperacillin and β -lactam antibiotics.

Similarly lactic acid, an effective antimicrobial agent, has shown enhanced activity when combined with copper. A study undertaken by Gyawali and Ibrahim [8] investigated the effect of adding low doses of copper to lactic acid to prevent growth of foodborne pathogens. Various combination treatments showed synergistic effect against *Salmonella* and *E. coli* O157:H7 on different food products. Copper has also been shown to exhibit a synergistic effect against biofilms of *Pseudomonas aeruginosa* when combined with quaternary ammonium cations [9]. Since transition metals exhibit antimicrobial properties, they have also been investigated for their combined effect. Silver has demonstrated enhanced antimicrobial effect against *E. coli* and *B. subtilis* when combined with other transition metals such as zinc, cobalt, cadmium, nickel and copper [10].

As stated in earlier chapters, transition metals have also been used to dope bioactive glasses for many biomedical applications. However, to date there has been no study undertaken on the antimicrobial effect of combining two or more metal oxides in a bioactive glass system. The aim of this chapter was to determine if cobalt, copper and/or zinc can work in combination and whether doping the phosphate based glass with synergistic combinations will retain the antimicrobial effect.

4.2 Aims

The aim of the given investigation was

- Investigate the effect of combining metal oxides such as cobalt, copper or zinc on growth and viability of *Escherichia coli* (NCTC 10538), *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 76615).

This was achieved by

- Evaluate the minimum inhibitory or bactericidal concentrations of 5 mol % cobalt, copper or zinc doped phosphate glass powders using broth microdilution assay.
- Determine the antimicrobial activity by checkerboard and time kill assays

4.3 Methods and Materials

4.3.1 Microbial strains

See section 3.3.1

4.3.2 Microbiological media

See section 3.3.2

4.3.3 Standard Growth curve

See section 3.3.3

4.3.4 Antimicrobial activity of 5 mol % cobalt, zinc and copper phosphate based glasses; alone and in combination against microorganisms in planktonic growth mode

4.3.4.1 Determination of Minimum inhibitory and bactericidal concentration using Broth Macrodilution

Broth macrodilution was performed in accordance with CLSI guidelines to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the glass particles. Double dilutions of each stock solution (100 mg/ml) were performed in the range from 50 – 0.2 mg/ml by adding 1 ml sterile PBS and 1 ml Mueller Hinton Broth (Oxoid Ltd, UK.). To maintain equal volumes throughout the procedure, 1 ml of solution was discarded from the last dilution. Triplicate samples were prepared in sterile Bixoux bottles (Thermo Scientific, UK.), inoculated with 10^5 CFU/ml and incubated overnight in shaking incubator at 200 rpm at 37 °C; *C. albicans* at 30 °C in an aerobic environment. Solution devoid of glass dissolution products, under the same growth condition, was used as a control. To avoid potential misinterpretation of turbidity due to coloured solution of glasses, liquid medium without microorganisms, but containing the same concentration of glass dissolution products were used as standard solutions for comparison.

After overnight incubation, the turbidity of the test solutions was checked against glass standard solutions. MIC was determined as the lowest concentration which showed no turbidity on visual inspection. One hundred μ l of the lowest concentration that was not visually turbid i.e. MIC and higher concentrations than MIC along with the controls were plated onto Mueller Hinton agar and incubated overnight at 37°C; *C. albicans* at 30°C on SDA in an aerobic environment. MBC was determined as the lowest concentration which yielded three log reductions i.e. a 99.9% reduction in CFU/ml compared to control. Tests were performed in triplicate and repeated three times.

4.3.4.2 Checkerboard assay to assess the antimicrobial efficacy in combination

To determine the effect of the glasses in combination, checkerboard assays were performed as explained here. To evaluate the effect of copper (Cu) and cobalt (Co), for instance, double dilutions of Cu dissolution products and Co dissolution products were prepared in MHB or SDB ranging from 50 – 0.2 mg/ml. For each microorganism 96 wells plates (Fisher Scientific, Leicester, UK) were set up, in duplicate, by adding 100 μ l of Cu glass dissolution products and 100 μ l of Co glass dissolution products. Thus, for each plate, the dissolution products of Cu were serially diluted along the ordinate and those of Co were diluted along the abscissa. Cu and Co glass dissolution products alone were set up as the controls, along with MHB or SDB. All wells were inoculated with 10 μ l of a 10^6 CFU/ml of test microorganism and incubated overnight in an aerobic environment at 37°C or 30°C as appropriate. Similar procedure was repeated with other combinations i.e. cobalt and zinc; copper and zinc.

Concentration of B (mg/ml)	Concentration of A (mg/ml)								
		25	12.5	6.25	3.13	1.56	0.78	0.39	0.0
	25								
	12.5								
	6.25								
	3.13								
	1.56								
	0.78								
	0.39								
	0.0								

Figure 4.1: shows schematic representation of checkerboard assay. Reducing concentrations of bioactive glass, A or B are added in rows and columns respectively. Ten μl of a 10^6 CFU/ml inoculum of each microorganism was added to determine synergism, indifference or antagonism.

The MIC of each metal alone and in combination was determined as the lowest concentration of metal dissolution product that completely inhibited the growth of the organism as detected with the naked eye. The effects of the combinations were then determined as synergistic, indifferent or antagonistic by calculating the fractional inhibitory concentration (FIC), and then the fractional inhibitory concentration indices (FICI) value for each combination [11].

$$\text{FIC} = \frac{\text{MIC of metal in combination}}{\text{MIC of metal alone}}$$

$$\text{FICI} = \text{FIC of metal A} + \text{FIC of metal B}$$

Where $\text{FICI} \leq 0.5$ shows synergism

$\text{FICI} > 0.5$ or < 4.0 shows indifference

$\text{FICI} > 4.0$ shows antagonism

4.3.4.3 Time kill assay to determine the antimicrobial efficacy in combination

The time kill of the glass dissolution products alone and in combination along with synergy determination was also assessed using a suspension method time-kill assay as described by White

et al. [11]. Three experimental suspensions were prepared at twice the MIC concentration, for each combination: Cu only suspension, Co only suspension and a combination Cu/Co suspension. All experimental tubes; each metal alone and in combination (Cu/Co, Cu/Zn and Co/Zn) were seeded with an initial microbial density of 10^5 cfu/ml. In addition, a control containing Mueller-Hinton broth seeded with a microbial inoculum and a control containing broth only, were prepared. All tubes were incubated aerobically at 37°C or 30°C for 24 hours in a shaking incubator at 200 rpm. At time periods, 0, 2, 6 and 24 hours, 100 µl aliquots were diluted 1:10 in D/E neutralization buffer to prevent antimicrobial carry-over. Diluted samples were sub-cultured on Mueller-Hinton agar and incubated overnight at 37°C or 30°C under aerobic conditions after which cfu were determined.

Synergy was defined as a $\geq 2 \log_{10}$ reduction in the colony count between the combination and the most active agent at 24 hour. Additive or indifference was a $< 2 \log_{10}$ decrease in the colony count at 24 hr by the combination compared with the most active single agent whereas antagonism was a $\geq 2 \log_{10}$ increase in colony after 24 hour between the combination and the most active agent [12-14].

4.3.5 Evaluation of antimicrobial activity of phosphate based glasses containing 50/50 mol % of metal oxides

The effect of glasses manufactured with 5 mol % of two different metal oxides was determined on the growth of microorganism studied. 5 mg/ml suspension of 5 mol % copper, cobalt, zinc alone and in combinations were prepared in relevant microbiological media. Each test sample was inoculated with 10^5 cfu/ml of the test microorganism. The samples were then incubated in an aerobic incubator at 37°C or 30°C with a shaking speed of 200 rpm, broth without glass discs served as negative control. At various time intervals; 0, 2, 6 and 24 hours, a 100 µl sample was taken out from each test container and serially diluted 1:10 in D/E neutralization buffer to

determine the viable count using spread plate method. All experiments were undertaken in triplicate. Since the pH of the aqueous media is likely to change upon glass dissolution therefore pH was also assessed at time 0, 2, 6 and 24 hours (See Appendix – Table A1).

Synergy was defined as a $\geq 2 \log_{10}$ reduction in the colony count between the combination and the most active agent at 24 hour. Additive or indifference was a $< 2 \log_{10}$ decrease in the colony count at 24 hr by the combination compared with the most active single agent whereas antagonism was a $\geq 2 \log_{10}$ increase in colony after 24 hour between the combination and the most active agent [12-14].

4.3.6 Statistical analysis

Two-way analysis of variance was carried out to determine statistical significances (GraphPad Prism). If a significant difference was detected a Tukey test was carried out to determine which values were significantly different. Differences were considered statistically significantly at a level of $P < 0.05$.

4.4 Results

4.4.1 Minimum Inhibitory and Bactericidal concentrations of cobalt, zinc and copper doped glasses using broth microdilution

The results demonstrated a greater antimicrobial activity of cobalt doped phosphate glass compared to zinc and copper doped phosphate glasses, as the MIC and MBC values are ≤ 3.13 mg/ml for cobalt, 1.5 – 6.25 mg/ml for zinc and 3.13 – 25 mg/ml for copper doped glass (Figure 4.2). *C. albicans* demonstrated reduced susceptibility to copper compared to the other microorganisms tested, where the highest concentration tested (25 mg/ml) of copper exhibited bactericidal effect.

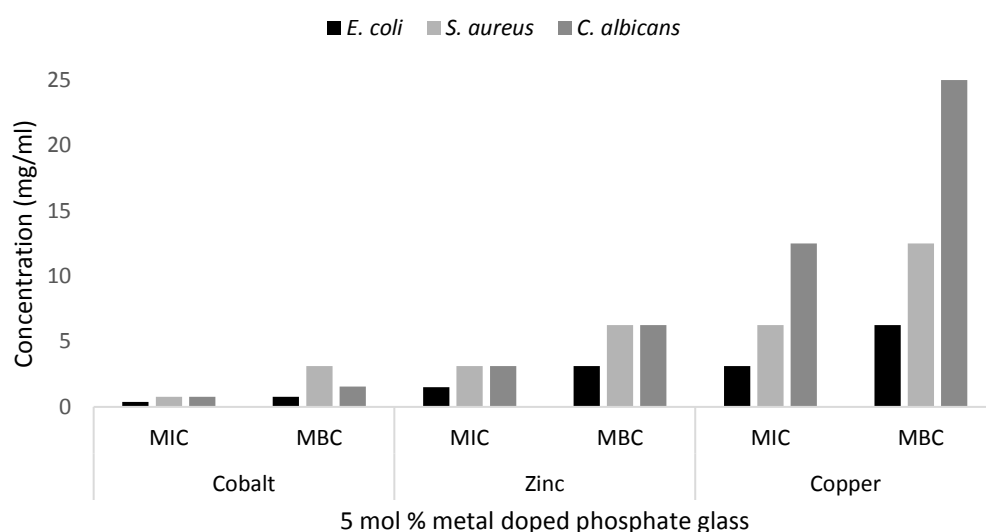


Figure 4.2: MICs and MBCs media value (N=3) of 5 mol % metal (cobalt, zinc, copper) doped phosphate glasses against *E. coli*, *S. aureus* and *C. albicans* determined using the broth microdilution assay.

4.4.2 Checkerboard assay to assess the antimicrobial efficacy in combination

All three combinations; cobalt and copper, cobalt and zinc, zinc and copper failed to demonstrate synergistic antimicrobial activity against the microorganisms tested except for cobalt and copper against *E. coli* with and FICI value of 0.5 (Table 4.1).

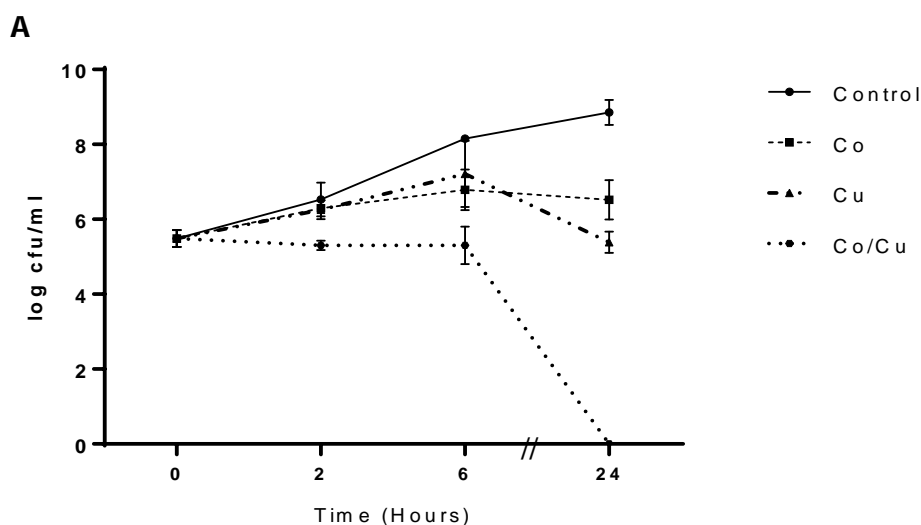
Microorganism	Combination tested	MIC alone/Combination	Mean FIC	Mean FICI	Result
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<i>E. coli</i>	Co/Cu	Co 3.13/0.78, 3.13/0.78	0.25, 0.25	0.5	Synergy
		Cu 12.50/3.13, 12.50/3.13	0.25, 0.25		
	Co/Zn	Co 3.13/1.56, 3.13/3.13	0.5, 1.0	1.75	Indifference
		Zn 6.25/6.25, 6.25/6.25	1.0, 1.0		
	Cu/Zn	Cu 12.50/12.50, 12.50/6.25	0.125, 0.5	1.375	Indifference
		Zn 6.25/3.13, 6.25/6.25	0.5, 1.0		
<i>S. aureus</i>	Co/Cu	Co 1.56/1.56, 1.56/0.78	1.0, 0.5	0.94	Indifference
		Cu 6.25/1.56, 6.25/0.78	0.25, 0.125		
	Co/Zn	Co 1.56/3.13, 1.56/3.13	2.0, 2.0	3.0	Indifference
		Zn 6.25/6.25, 6.25/6.25	1.0, 1.0		
	Cu/Zn	Cu 6.25/6.25, 6.25/1.56	1.0, 0.25	1.87	Indifference
		Zn 6.25/12.5, 6.25/1.56	2.0, 0.25		
<i>C. albicans</i>	Co/Cu	Co 3.13/1.56, 6.25/3.13	0.5, 0.25	1.0	Indifference
		Cu 12.5/6.25, 25.0/6.25	0.25, 0.25		
	Co/Zn	Co 3.13/1.56, 3.13/1.56	0.5, 0.5	2.0	Indifference
		Zn 6.25/12.5, 12.5/12.5	2.0, 1.0		
	Cu/Zn	Cu 12.5/6.25, 25.0/6.25	0.5, 0.25	0.95	Indifference
		Zn 3.13/3.13, 12.5/3.13	1.0, 0.25		

Table 4.1: Antimicrobial efficacy of 5 mol % cobalt, zinc and copper doped phosphate based glasses against planktonic growth of *E. coli*, *S. aureus* and *C. albicans*, using a checkerboard assay. The results are shown in duplicate and expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), where $FICI \leq 0.5$ shows synergy, $FICI > 0.5$ or ≤ 4.0 shows indifference, and $FICI > 4.0$ shows antagonism.

4.4.3 Evaluation of synergistic effect of glasses using time kill assay

Time kill curves of 5 mol % cobalt, zinc or copper doped phosphate glasses alone and in combination against *E. coli* are shown in Figure 4.3. A significant antimicrobial effect of the glasses tested and their combinations was not observed until 6 hours. At 6 hours cobalt showed a significant reduction ($p = 0.0089$) in the bacterial count compared to copper and zinc doped glasses. Similarly, significant results were observed for all three glass combinations at 6 hours ($p < 0.0001$). At 24 hours, the antimicrobial effect of cobalt and copper was enhanced ($p < 0.0001$) while zinc failed to exhibit antimicrobial effect. When considering the effect of combining two different glasses, a synergistic activity between Co/Cu, Co/Zn and Cu/Zn was observed, as there is a visible reduction in colony count at 24 hours below single agents.



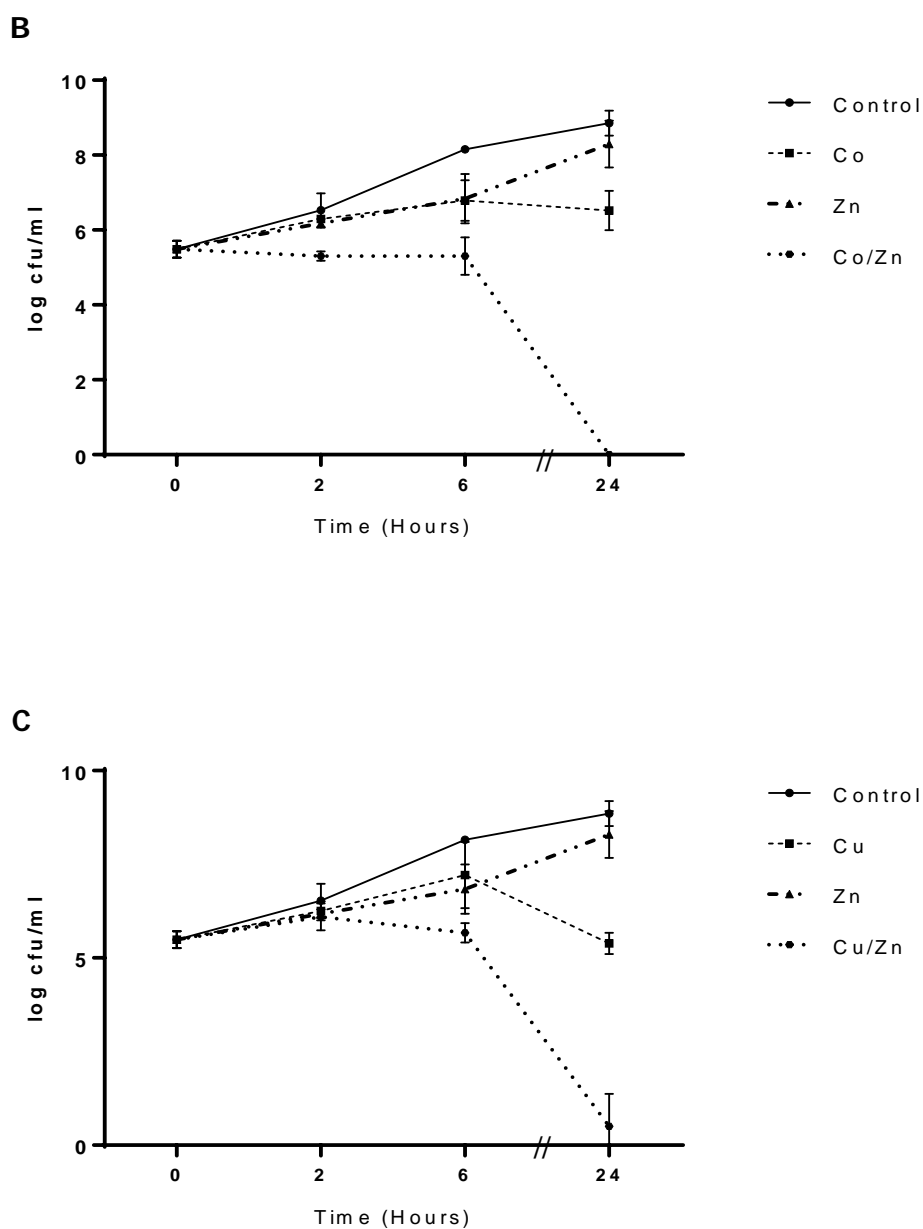
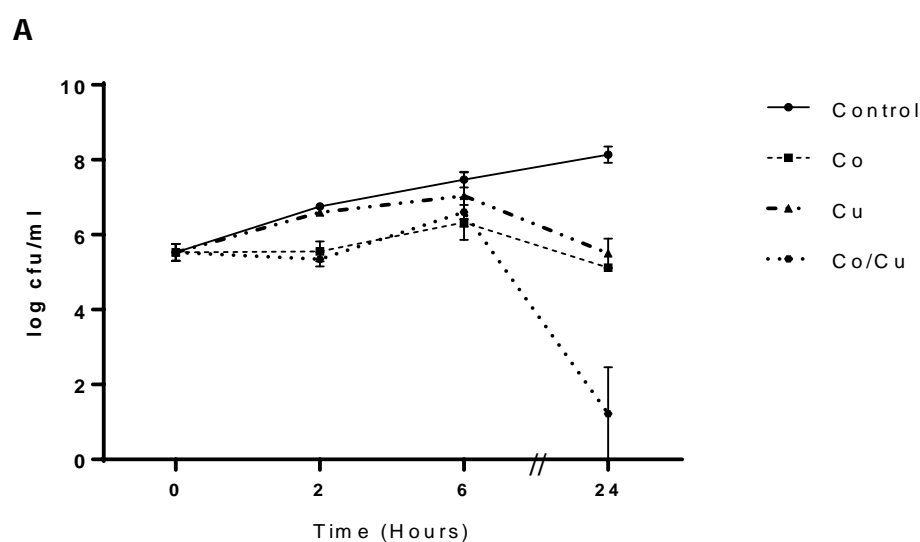


Figure 4.3: shows time kill curves of 5 mol % cobalt, zinc and copper doped phosphate glass powders alone and in combination, at 2×MIC concentration, against *E. coli*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean \pm SD (N=3).

The antimicrobial effect of 5 mol % cobalt, copper or zinc doped glasses against *S. aureus* is shown in the figure 4.4. A non-significant decrease in bacterial count was seen at 2 hours, and at 6 hours except for cobalt doped glass and Co/Zn glass combination. Whilst significant activity was observed at 24 hours by copper and cobalt against *S. aureus* zinc failed to exhibit antimicrobial activity. While all glass combinations exhibited a synergistic effect against *E. coli*, only two combinations i.e. Co/Cu and Co/Zn showed synergistic effect against *S. aureus* (Figure 4.3). A significant difference ($p < 0.001$) in the bacterial count was observed at 24 hours by cobalt, copper, Co/Cu and Co/Zn when compared to *S. aureus* control.



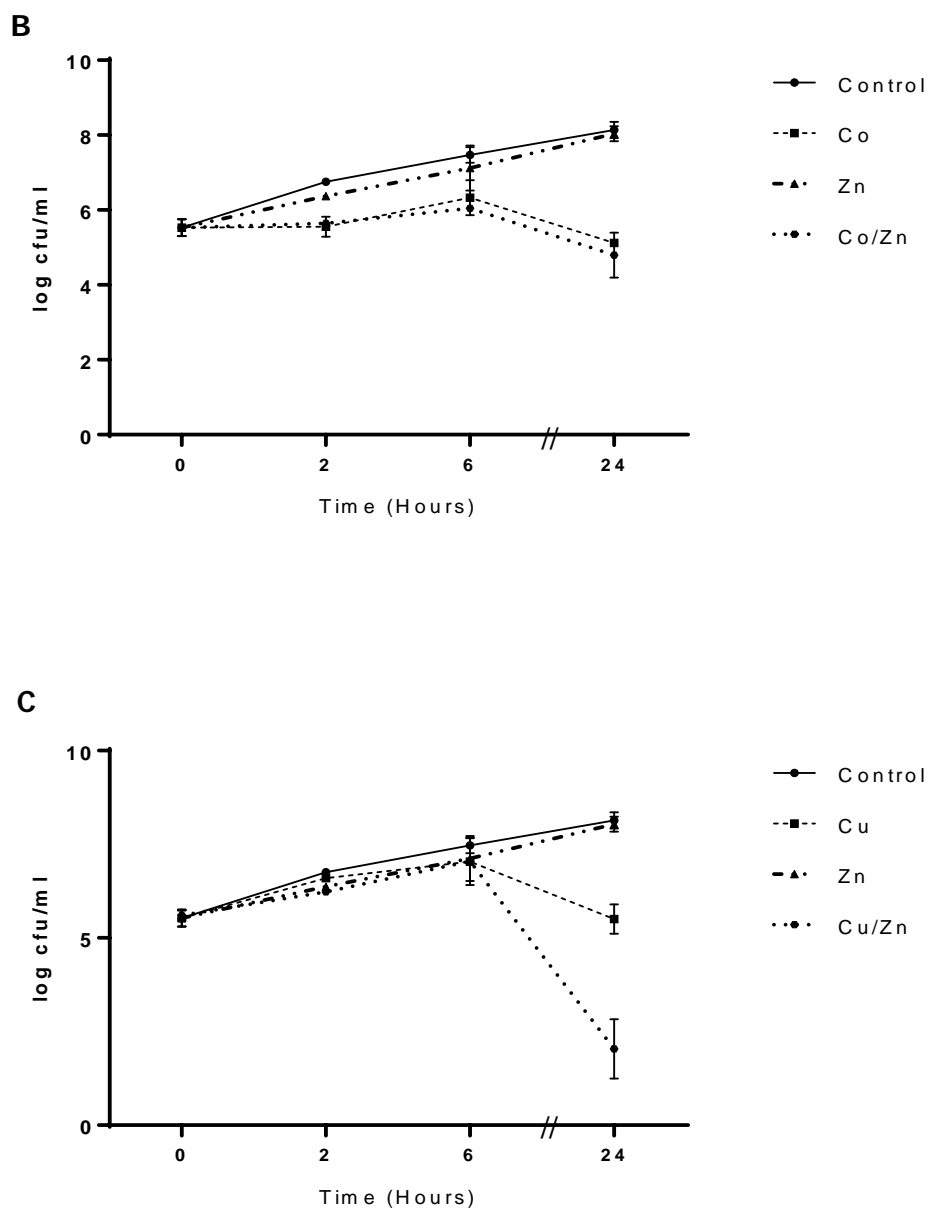
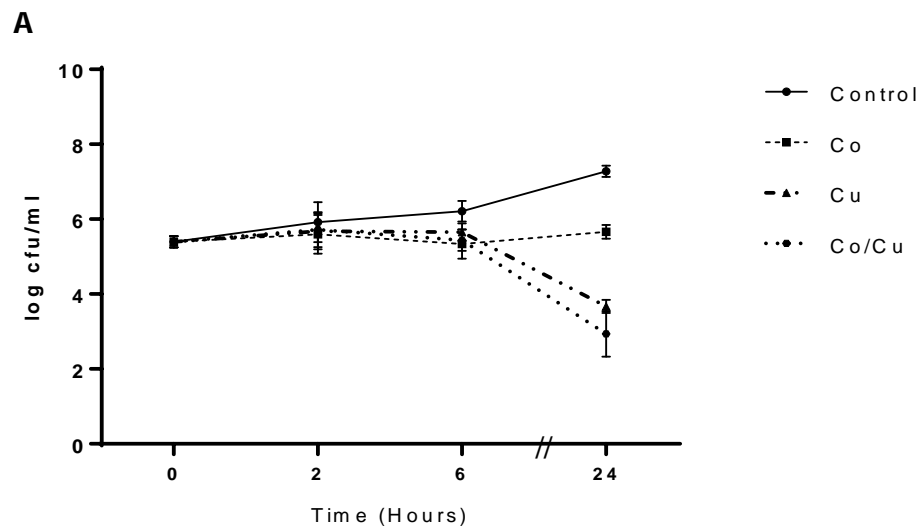


Figure 4.4: shows time kill curves of 5 mol % cobalt, zinc and copper doped phosphate glass powders alone and in combination, at $2\times$ MIC concentration, against *S. aureus*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean \pm SD (N=3).

The antimicrobial effect of 5 mol % cobalt, copper or zinc doped glasses against *C. albicans* is shown in the figure 4.5. A non-significant decrease in microbial count was seen at 2 and 6 hours. A significant decrease in colony count was observed at 24 hours by copper ($p < 0.001$), cobalt ($p = 0.002$) and zinc ($p = 0.0298$) when compared to non-treated cultures (control). Similarly, combinations tested showed significant antimicrobial effect ($p < 0.001$). Whilst significant activity was observed at 24 hours by glasses alone and combinations, synergistic effect was not seen.



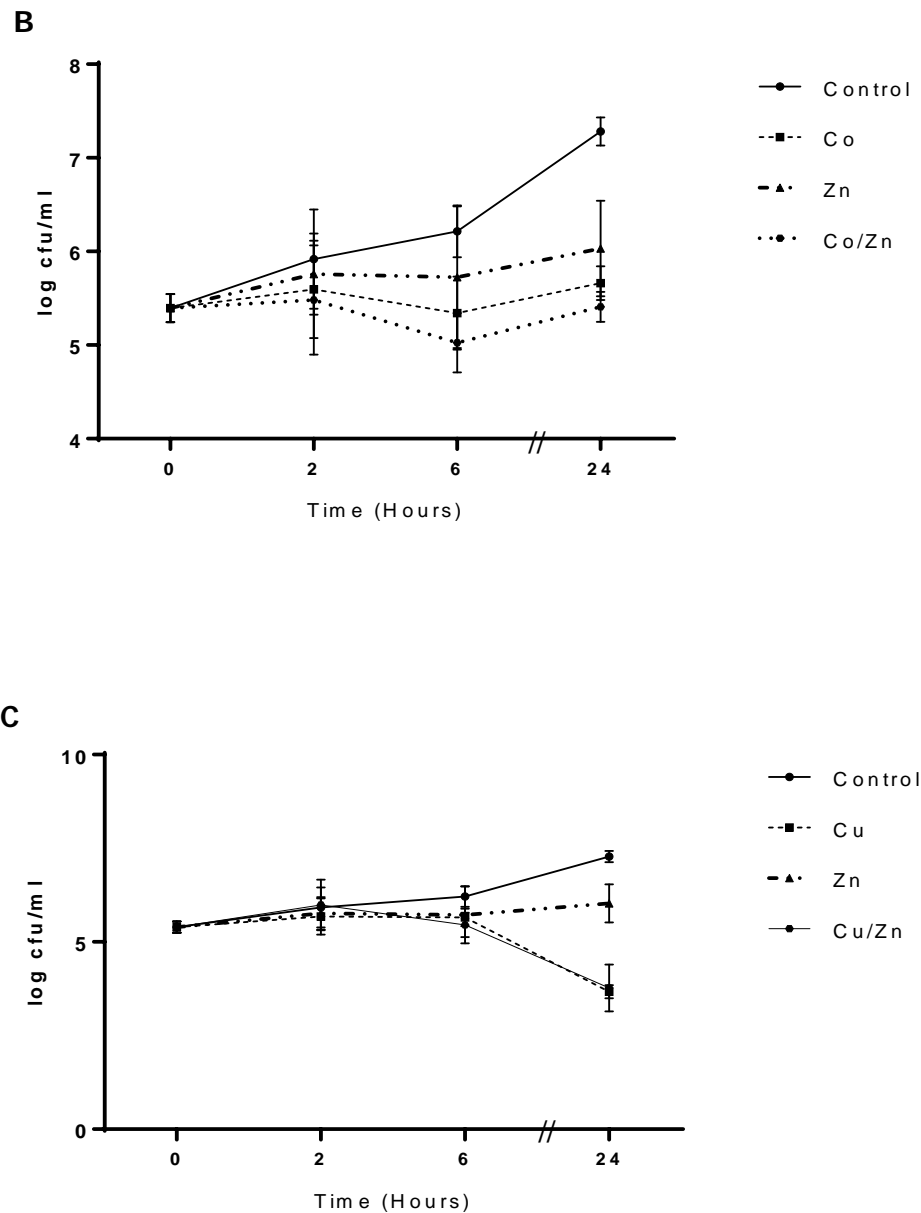
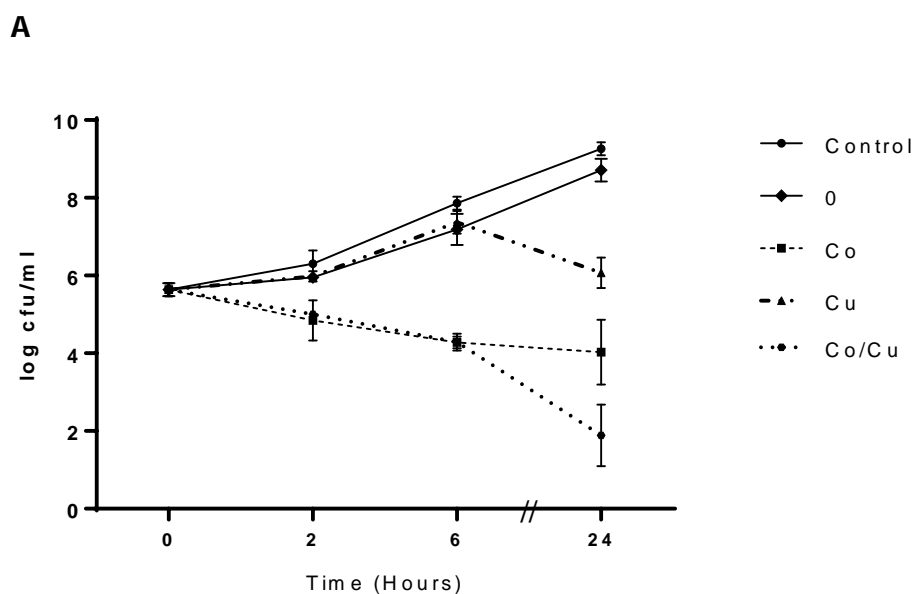


Figure 4.5: shows time kill curves of 5 mol % cobalt, zinc and copper doped phosphate glass powders and in combination, at 2×MIC concentration, against *C. albicans*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean ± SD (N=3).

4.4.4 Evaluation of antimicrobial activity of phosphate based glasses containing 50/50 mol % of metal oxides

Time kill curves of cobalt, copper and zinc doped glasses alone and in combination (50/50 mol %) against *E. coli* are shown in Figure 4.6. A significant antimicrobial effect was seen for cobalt at 6 and 24 hours when compared to untreated control ($p < 0.0001$) and un-doped phosphate based glass ($p < 0.0001$), whereas copper and zinc failed to demonstrate a significant decrease in microbial count. When considering the effect of combining two different glasses (5 mol % of each dopant), a synergistic activity was only seen for Cu/Zn, as there is > 2 log reduction in colony count at 24 hours compared to copper and zinc alone.



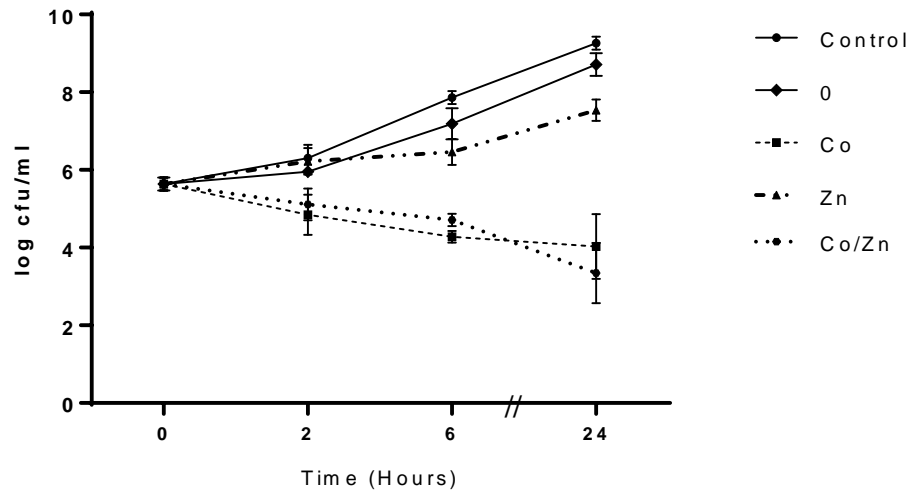
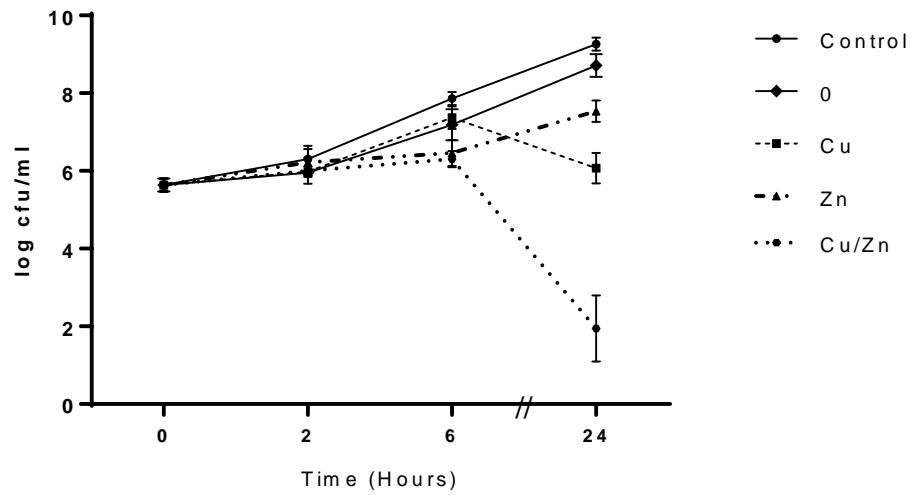
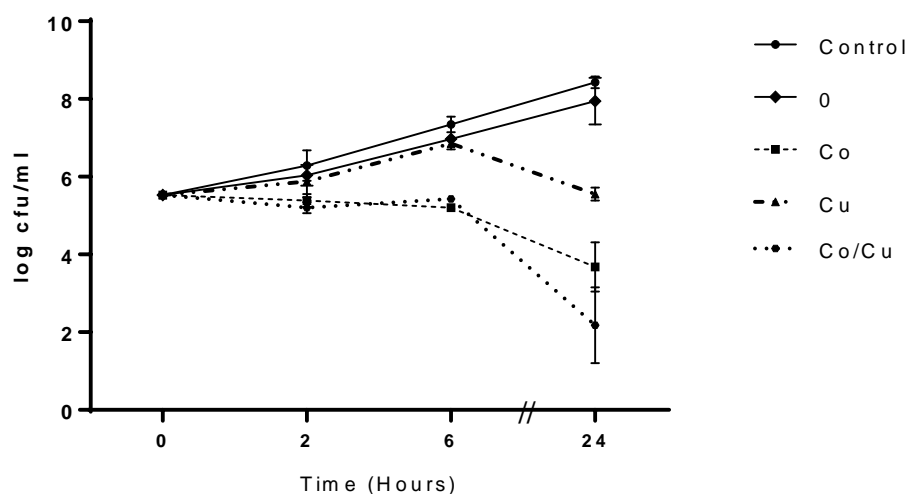
B**C**

Figure 4.6: shows time kill curves of 50/50 mol % cobalt, zinc and copper doped phosphate glass powders alone and in combination against *E. coli*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean \pm SD (N=3).

Figure 4.7 shows the antimicrobial effect of combining two metal oxides in the given phosphate glass system against *S. aureus*. A significant decrease in bacterial count was seen at 2 hours for 5 mol % cobalt doped glass ($p = 0.0376$), whereas a non-significant difference was observed for 5 mol % copper or zinc doped glasses demonstrating lack of antimicrobial activity. Similar effect was seen at 6 and 24 hours. The combined effect of glasses showed Cu/Zn have synergistic effect against *S. aureus* whereas Co/Cu and Co/Zn showed indifference.

A



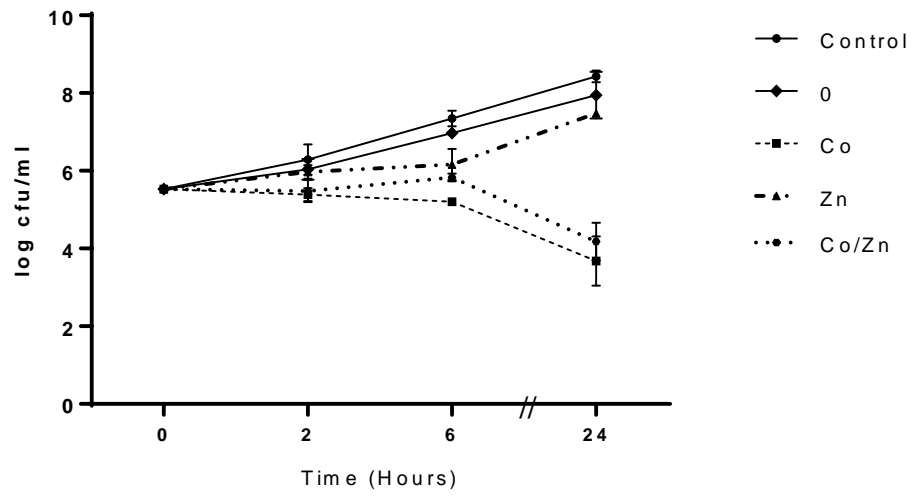
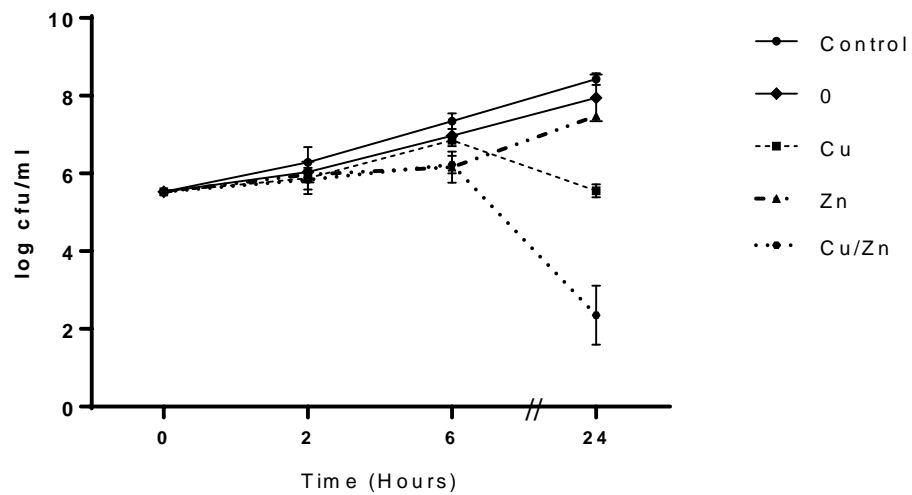
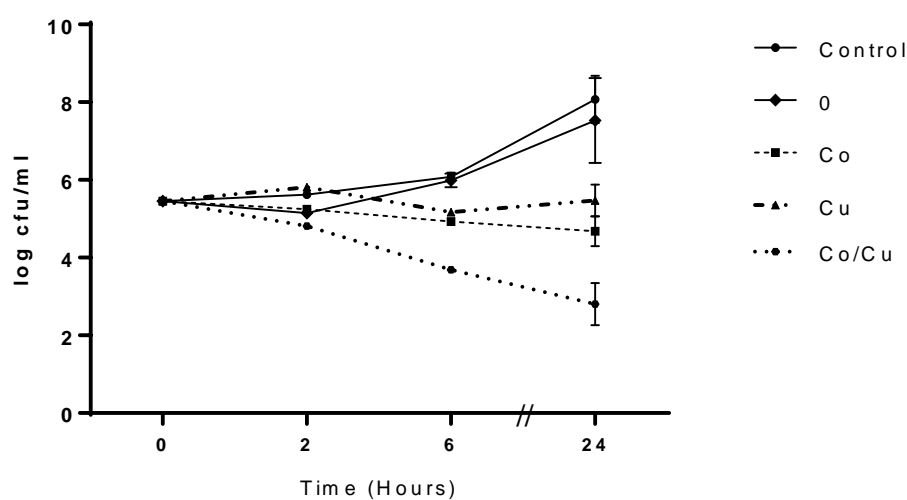
B**C**

Figure 4.7: shows time kill curves of 50/50 mol % cobalt, zinc and copper doped phosphate glass powders alone and in combination against *S. aureus*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean \pm SD (N=3).

Figure 4.8 shows the time kill curves of cobalt, copper or zinc doped phosphate based glasses; alone and in combination against *C. albicans*. Significant decrease in growth of *C. albicans* was seen at 24 hours when treated with cobalt, copper or zinc doped glasses alone ($p < 0.0001$). However, the antimicrobial effect was less pronounced against *C. albicans* compared to *E. coli* or *S. aureus* (Figure 4.5 – 4.6). Combination of Co/Cu resulted in synergistic activity for *C. albicans*, whereas ‘Indifference’ was observed for Co/Zn and Cu/Zn combinations (Figure 4.7).

A



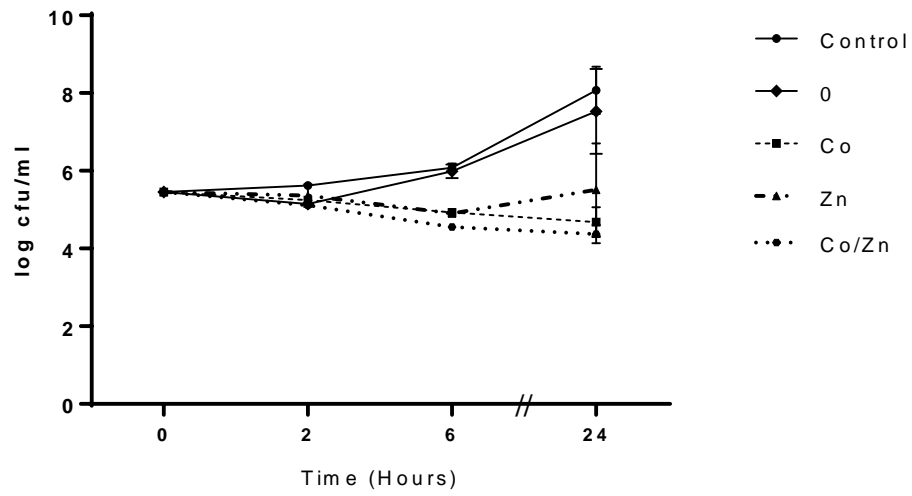
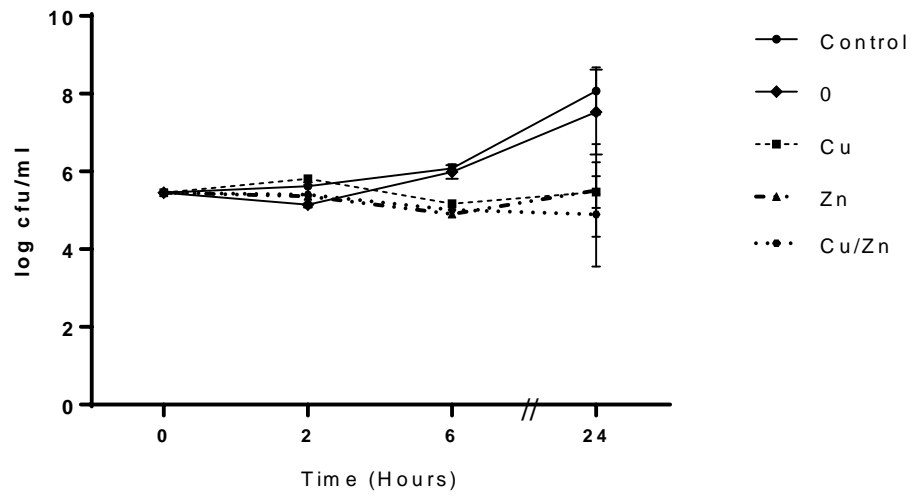
B**C**

Figure 4.8: shows time kill curves of 50/50 mol % cobalt, zinc and copper doped phosphate glass powders alone and in combination against *C. albicans*. The combinations investigated were (A) Co/Cu, (B) Co/Zn and (C) Cu/Zn. Microbial viability is presented as log CFU/ml. Data shown are expressed as mean \pm SD (N=3).

4.5 Discussion

The polymicrobial nature of various infections and development of resistant strains over the past few decades have become a huge clinical problem. Therefore, researchers are striving not only to gain comprehensive knowledge about drug resistant strains but also assessing the alternative treatments such as combination prospects. This study not only adds to the current knowledge of antimicrobial potential of bioactive glasses but also provides an insight to the effect of combining metal oxides within a glass system. The purpose of this chapter was to identify metal doped glass compositions with synergistic antimicrobial effect against microorganisms such as *E. coli*, *S. aureus* and/or *C. albicans*.

Five mol % cobalt, copper or zinc doped phosphate glasses were tested alone and in combination against clinically relevant microorganism. The synergistic effect was studied using two different methods; checkerboard and time kill assays to attain comprehensive results. The results of this study demonstrated that 5 mol % cobalt, copper or zinc doped phosphate glass systems possess antibacterial and antifungal activity proving effective against *E. coli*, *S. aureus* and *C. albicans* in planktonic form. The lack of antimicrobial activity seen for un-doped phosphate based glass demonstrates that the antimicrobial efficacy of the doped glasses is derived from the cobalt, copper or zinc ions. Additionally, this study is the first to report a synergistic antimicrobial effect of metal oxides against *E. coli*, *S. aureus* and *C. albicans* (discussed below).

Variable results were seen with both methods, most combinations showed indifference in checkerboard except for Co/Cu against *E. coli*, Time kill analysis was performed using two slightly different methods. In method 1 (Time Kill 1 – TK1), conventional assay was used where single metal oxide doped glasses were separately used to test synergy. Whereas, in method 2 (Time kill 2 – TK2), glasses were prepared with a combination of two metal oxides within the same glass system. Whilst no synergistic effect was seen against *C. albicans*, in TK1, Co/Cu and

Co/Zn showed a synergetic antimicrobial effect against *E. coli* and *S. aureus* whereas Cu/Zn showed only against *E. coli*. On the other hand, in TK2, Cu/Co and Cu/Zn showed synergistic effect against *E. coli* whereas, only Cu/Zn against *S. aureus* with no synergism against *C. albicans*.

A considerable variation in the combination results was observed when checkerboard and time kill results were compared, nonetheless there was no major categoric difference i.e. from synergism to antagonism or vice versa. The difference in the data could be attributed to the methodologies used. In checkerboard studies, the microtiter plates were incubated in static incubator and the results demonstrated an 'indifference' whereas time kill studies were undertaken in a shaker at 200 rpm and few combinations showed synergism. Unlike checkerboard assay (static conditions), in time kill studies metal ions are more likely to come in contact with microorganisms due to continuous agitation and penetrate cells therefore exhibiting an enhanced antimicrobial effect. Furthermore, in checkerboard assay the slightly coloured solution of glasses makes it difficult to interpret the visible growth and could have contributed to incorrect FICI calculations.

Several studies have been undertaken to compare the accuracy and sensitivity of the two methods. Petrou & Rogers conducted a comparative study on the efficacy of polyene and azole combinations against fungal strains and noted better sensitivity and reproducibility with time kill analysis [15]. Similarly, Lewis *et al.*, indicated that E-test and time kill assays yield better results compared to checkerboard tests [16]. The rates of synergy detected by time kill assay on MDR *Acinetobacter baumannii* were higher than the rates detected by checkerboard assay [17]. A systematic review conducted by Zusman *et al.*, concluded that synergy rates are higher in studies using time kill method than the checkerboard or E-test method [18]. Thus, the data presented here on combination analysis using time kill assays is likely to be more accurate.

Time kill assays were performed using two different approaches. In method 1 glasses prepared with one metal oxide were combined to evaluate synergistic potential whereas for method 2, two metal oxides were incorporated within the glass system at 5 mol % ratio of each component. The results from the two methods showed a slight variation for *E. coli* and *S. aureus* however no synergism was detected against *C. albicans*. All combinations demonstrated a synergistic antimicrobial effect using method 1 but only Cu/Zn showed synergism in method 2. The lack of synergism in method 2 compared to method 1 could be attributed to the glass dissolution behaviour. As discussed in section 2.5, glass dissolution is a complex process which depends on several factors such as glass composition, structure, dissolving media, pH and temperature. The incorporation of an additional metal oxide in the glass system is likely to change the structure of the glass network and subsequently the dissolution behaviour and ion release profile. Furthermore, microbiological media such as nutrient broth provides a complex environment with a number of ionic species that may reduce the concentration of a free metal ion by precipitation or by the formation of soluble complexes [19]. Therefore, a direct comparison between the two methods cannot be made due to different release profile of the antimicrobial ions from glasses.

Despite the variation in time kill results from two methods, the present study has established that metal doped bioactive glasses can be used in combination as they possess a synergistic antimicrobial activity. Some drug combinations with different modes of actions can produce synergistic effect against multidrug resistant strains. Bioactive glasses are generally believed to have a broad spectrum antimicrobial action; however, various metal ions have also been shown to target specific cell component or process. For instance, copper ions are believed to target the cell membrane [20] whereas cell death due to cobalt ions is brought about due to hypoxic conditions [21, 22]. Nonetheless, the full mechanism of action of combining metal oxides is not yet fully understood.

In line with the results of chapter 3, the synergistic antimicrobial action appeared organism specific, with *E. coli* being the most sensitive whereas *C. albicans* was least susceptible. This could be attributed to the outer cell wall structure of the strains tested. Gram negative bacteria unlike Gram positive bacteria have ion channels that allow penetration of metallic ions through the outer membrane which subsequently bring about cell death. Furthermore, in addition to thick peptidoglycan, cationic sequestering due to anionic metal binding sites occurs on the surface of Gram positive bacteria which contributes to resistance of *S. aureus* killing [23-26]

4.6 Conclusion

Five mol % cobalt and copper doped phosphate glass demonstrated an antimicrobial activity against Gram positive and negative bacteria, as well as *C. albicans*. Combination studies using time kill assays showed synergism. The synergistic antimicrobial efficacy possessed by Cu/Co and Cu/Zn against *E. coli* and Cu/Zn against *S. aureus* may be used to coat/impregnate urinary catheters for preventing device related infections. A better understanding of the active concentration of metallic ions needed to kill microbes can be achieved by performing elemental analysis.

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Chapter 5

Cytotoxic evaluation of cobalt or zinc doped bioactive glasses with human uroepithelial cells

5.1 Introduction

Urinary catheters, part of the catheter coated with bioactive glasses or glass beads injected in to the bladder lining will come in contact with body environment and therefore should be biocompatible. Bioactive glasses have been extensively investigated for bone repair and regeneration as well as in dental field. Biocompatibility of bioactive glasses is a key element when it comes to their *in vivo* applications. The composition of bioactive glasses has a significant effect on their ability to support cell growth and proliferation. For instance, calcium ions in Bioglass® play a key role in stimulating osteoblast-like cell proliferation and differentiation. It is therefore important that glasses should release an optimum level of ions which are sufficient to elicit a desired response with minimal detrimental effects to the living tissues. Several studies have found bio-glasses to be safe materials that allow adhesion, proliferation and differentiation of cells [1-7].

Several phosphate glass systems have been developed and studied for their biocompatibility. In a study conducted by Uo *et al.*, cytocompatibility of a phosphate glass system (P_2O_5 -CaO- Na_2O) was evaluated against dental pulp cells. It was found that the glass was not very cytotoxic when P_2O_5 content was 50% or below, whereas glasses with P_2O_5 content greater than 50% were rendered cytotoxic due to high pH [8]. Similar findings were reported by Navarro *et al.* that showed a non-toxic effect of a titanium doped P_2O_5 -CaO- Na_2O system on Saos-2 cells [9]. Salih *et al.*, studied the biological response of a similar ternary glass system against two human osteoblast cell lines; MG63 and HOS and showed enhanced cell growth with dissolution products of the glasses studied [10]. Biocompatibility studies of sodium phosphate glasses showed low levels of peroxide and interleukin- 1β released by macrophages which maintained a round non-activated morphology. The study established that the glass produced a very low inflammatory response [3].

In vivo studies have also been conducted on bioactive glasses to investigate bonding of bioglass to bone. The glasses have been implanted not only in mice but also in mammals such as monkeys, dogs and baboons with no evidence of toxicity [11-14]. Several studies have been performed to understand the inflammatory response of the body to bioactive glasses. Walker *et al.*, reported the effect of injecting Bioglass®, in bladder tissues of rabbits, to correct urinary incontinence. The surrounding tissue was found not to contain increased calcium and silicon, no histological changes and inflammatory response were observed [15]. Rectenwald and co-workers injected glass intraperitoneally into mice and found it attenuated a proinflammatory response due to endotoxemia [16]. Similarly bioactive glasses containing CaO–SiO₂–P₂O₅ have been shown to bond with hard and soft tissue without causing local or systemic toxicity and any signs of inflammation [17, 18].

Whilst a growing body of research is available on lack of toxicity of bioactive glasses, nonetheless a few studies have reported detrimental effects on living tissue. For instance, Luo *et al.*, conducted *in vitro* analysis of cytotoxicity of silver containing borate glasses and found the glasses had toxic effects on cell viability [19]. In some other studies, cytotoxicity of borate glasses has been shown, however this has been attributed to be a consequence of high pH due to the faster degradation rate of the borate glasses [20-22].

From the above-mentioned studies it is evident that glasses are safe for use *in vivo*, however a large number of the studies have been conducted on osteoblasts and limited or no data is available on the effect of bioactive glasses on epithelial cells such as uroepithelium. Since the aim of the research is to develop novel glass coatings or glass beads that can be injected in to the bladder lining to treat urinary tract infections, hence the cytotoxic effect of the given glasses needs to be evaluated on the cells lining the urinary tract. Therefore, the focus of the present work was to

investigate the cytotoxic effects, if any, of the cobalt or zinc doped glasses on human epithelial cells.

The urinary tract is lined by transitional epithelium which is a type of tissue containing multiple layers of epithelial cells. The tissue is found in renal pelvis, urinary bladder, ureters and urethra. Therefore, for this study normal human uroepithelial cells (HUCs) and a cancer cell line (T24) were used. These cells exhibit a characteristic cobblestone morphology in culture (Figure 5.1).

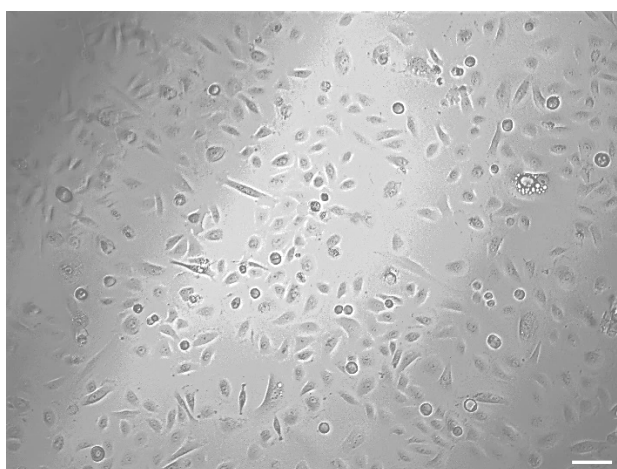


Figure 5.1a: Normal human uroepithelial cells. Scale bar indicates 100 μm .

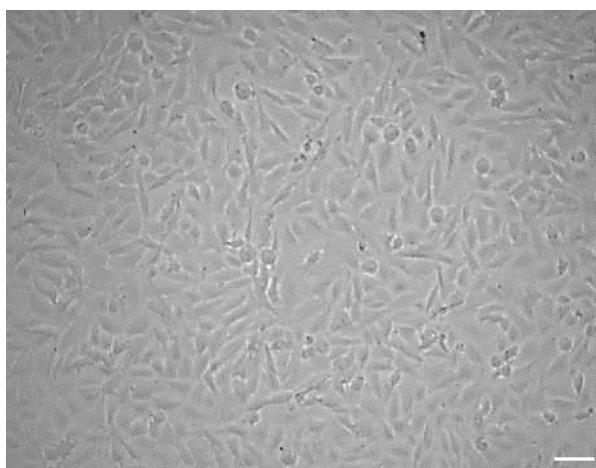


Figure 5.1b: Bladder cancer cells – T24. Scale bar indicates 100 μm .

5.2 Aims

The aims of this study were to:

- Investigate the cytotoxic effects of cobalt and zinc doped phosphate based bio-glasses (0, 3, 5 and 10% mol) on uroepithelial cells; normal human uroepithelial cells (HUC) and transitional carcinoma cells (T24).

This was achieved by studying:

- The effect of dissolution products of cobalt and zinc doped glasses on HUC and T24 cells
 - The effect of pH buffering
- The effect on viability of cells when in direct contact with cobalt and zinc doped glasses.

5.3 Methods and Materials

5.3.1 Glass preparation

See section 2.3.1

5.3.2 Cell culture

All cell culture work was undertaken in a class II biosafety cabinet with HEPA filtration units. Before and after use, the cabinet was cleaned with 70% (v/v) ethanol (Fisher Scientific, UK) and UV light was also used to disinfect the cabinet prior to use each day. Once every two weeks, the cabinet and incubators were cleaned with 10% Distel (Fisher Scientific, UK). Waste media and cells were disposed of using Virkon[®] (Sigma Aldrich, Dorset, UK).

5.3.1.1 Maintenance of Human uroepithelial cells

Human uroepithelial cells (HUC) were purchased from ScienCell laboratories, USA and cultured in a T75 flask (Fisher Scientific, UK) coated with poly L-lysine (ScienCell laboratories) at concentration of 2µg/cm². Before seeding cells, flasks were rinsed twice with sterile water and maintained in recommended uroepithelial cell media (UCM, ScienCell laboratories) supplemented with uroepithelial cell growth supplement (UCGS, ScienCell laboratories) and 1% penicillin/streptomycin (Fisher Scientific, UK) at 37°C and 5% CO₂ incubator. The media was changed every three days until the culture reached 70% confluency, thereafter the media was changed every other day until the culture was approximately 90% confluent.

Once 80- 90% confluent, cells were subcultured. First cells were washed twice with sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma Aldrich, Dorset, UK). To harvest cells 8 ml of sterile DPBS was added to the flask along with 2 ml of 0.25% trypsin/EDTA solution (ScienCell laboratories) and incubated at 37°C for 2 minutes. At the end of incubation, the flask was gently tapped to dislodge cells. The contents of the flask were then transferred into a centrifuge tube containing 5 ml of fetal bovine serum (FBS). Ten ml of trypsin neutralising

solution (ScienCell laboratories) was used to collect remaining cells from the flask and transferred into the centrifuge tube. Cells were then centrifuged at 1000 rpm for 5 minutes and resuspended in culture medium and seeded into a new flask and/or used for experiments.

5.3.1.2 Maintenance of T24 cells – Bladder transitional cell carcinoma

T24 (ATCC® HTB-4™) the human bladder carcinoma cells were purchased from the American Tissue Culture Collection and cultured in McCoy's 5A medium (ATCC) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere of 5% CO₂ and at 37°C. When 80 - 90% confluent cells were harvested using 0.25% trypsin, 0.03% EDTA solution. Culture media was removed, cells were washed with sterile DPBS (Sigma Aldrich, Dorset, UK) and 2 ml of trypsin-EDTA solution was added. The flask was incubated at 37°C for one minute. Following incubation 3 ml of complete media were added to neutralise the effect of trypsin-EDTA solution. The contents of the flask were added in a 15 ml centrifuge tube and spun at 1000 rpm for 3 minutes to harvest cells. After removing the supernant, the pellet was re-suspended in 1 ml of culture media and the cell count was performed as explained in section 4.3.2.3. The cells were then seeded into new flasks at a density of 1000 cells/cm² or used for experiments.

5.3.1.3 Viable cell counts using the trypan blue exclusion method

The viability of the cells in suspension was performed using the trypan blue exclusion method. The technique is based on the fact that viable cells have intact cell membranes whereas the dead cells have damaged i.e. perforated cell membranes which allows penetration of certain dyes such as trypan blue. Therefore, dead cells take up the dye and appear blue when looked under light microscope, whereas live cells have clear cell body.

To perform a viable cell count, 10 µl of cell suspension was added in 40 µl of trypan blue solution (Fisher Scientific, UK). The suspension was mixed thoroughly and loaded onto a haemocytometer (Fisher Scientific, Leicester, UK). Live cells were counted in four sets of 16 corner squares and number of viable cells were counted as under:

$$\text{Viable cells/ml} = \text{Average cell count} \times 10,000 \times 5$$

(Multiplied by 5 to correct for 1:5 dilution from trypan blue addition).

5.3.2 Determination of cytotoxic effect of cobalt doped and zinc doped phosphate based glasses

5.3.2.1 Direct contact

Direct contact assay allows both qualitative as well as quantitative assessment of cytotoxicity. The test material is placed on the cultured mammalian cells *in vitro* and the cytotoxic effect can be evaluated by observing the changes in the cell morphology and by quantifying the number of viable cells after exposure to the test material. The test was performed in accordance with ISO-10993-5 for testing in vitro cytotoxicity of medical devices [23]. To evaluate the effect of cobalt doped or zinc doped phosphate glasses cells were cultured in 6 wells plate. A known aliquot of continuously stirred cell suspension was added in each well and cells were evenly distributed by gentle horizontal rotation of the plates. Cells were then incubated in a humidified atmosphere of 5% CO₂ and at 37°C until the culture had grown to 90 – 100% confluency.

Before placing glass discs on the cells monolayer, cell morphology and confluency was verified under the light microscope. Culture medium was removed and 2 ml of fresh culture media was added. Heat sterilised glass discs (Ø 10 mm) were then placed on the cell layer, care was taken to avoid mechanical damage to cells while placing samples, in triplicates. It was ensured that the glass discs cover approximately 1/30th of the cell monolayer. Following 24, 48 and 72 hours

incubation at optimum conditions cell viability was performed using MTT reagent as described in 5.3.3.2.

5.3.2.2 Indirect contact

Medical devices when *in vivo* are subject to degradation, therefore it is vital to evaluate the cytotoxic effect of the dissolution products of the test specimen on the growth and proliferation of mammalian cells. To test the cytotoxic effect of dissolution products, conditioned media was prepared as described in ISO-10993-5. Sterile glass discs were incubated in 25 ml of respective complete cell media at 37°C for 24 hours. Since phosphate glasses have uniform dissolution over a period and the glass products are likely to get flushed out of the body within 24 hours, hence the time chosen for the extraction of glass products. After 24 hours incubation, conditioned media was filtered through 0.2µ Ministart syringe filter (Sartorius Biotech, Germany) and pH readings were taken using an accumet® pH meter (Fisher Scientific, UK). The conditioned media from each sample was then divided in two parts; one set of the media was not neutralised whereas the other was neutralised. The pH neutralisation was achieved by keeping the universals in 5% CO₂ incubator for one hour. pH readings were recorded before and after incubation.

Cells were harvested and seeded, in quadruplicate, into 96 wells plates at a density of 6×10^3 cells per well in 100 µl media. The plates were then incubated overnight to allow cells to attach. Following incubation, media was removed and cells were treated with 100 µl conditioned media for 24, 48 and 72 hours. Two plates were prepared for each time point, one was treated with conditioned media without pH buffering and the other treated with pH buffered media. During incubation, cells were treated with fresh conditioned media every 24 hours. Ethanol was used as positive control whereas cells grown in complete cell media served as negative control. At each time point cell viability was determined using MTT test as described below and the treatments were cytotoxic if the cell viability was less than 70% compared to the negative control.

An MTT viability assay (Thermo Fisher, UK) was used as to determine the cytotoxic effect of glass dissolution products. The assay works on the principle that metabolically active cells have NAD(P)H dependent cellular oxidoreductase enzymes which reduces the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) into insoluble formazan crystals. The crystals can be solubilised using detergents such as SDS or DMSO and the optical density of the solution gives direct measure of cell viability. A 12 mM stock solution of 3 - (4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) was prepared by adding 1 ml PBS to 5 mg MTT reagent. At each time point, conditioned media was removed from each well and MTT reagent, diluted 1/10 in phenol free media, was added to the cells. The plates were then incubated in the dark for 3 hours at 37°C/5% CO₂. Following incubation, the solution was removed from the wells and 100 µl of DMSO (Sigma – Aldrich, UK) was added to each well and left to incubate at 37°C for 10 minutes. The optical density was measured using an Ascent MultiScan GO spectrophotometer (Fisher Scientific, Leicester, UK) at 570 nm (test) and 675 nm (reference).

5.3.2.3 Cytotoxic evaluation using Live/Dead staining

To evaluate the cytotoxic effect of conditioned media on cells, live/dead staining was performed. Conditioned media was prepared as explained in section 4.3.3.2. A cell suspension containing 10,000 cells in complete media was added in each well of a 24 well plate. Cells were incubated overnight to allow adherence of the cells onto the well surface. Following incubation, media was discarded and cells were treated with conditioned media for 24, 48 and 72 hours. At each time point, a positive control was established by incubating cells with 70% ethanol for 45 minutes. Cells treated with complete media served as negative control. 100 µl staining solution containing 0.5 µM calcein-green and 2.5 µM ethidium homodimer - 1 was added to T24 cells and 2.0 µM calcein-green and 2.5 µM ethidium homodimer-1 was added to HUC. The cells along with staining solution was incubated for 45 minutes. Fluorescence microscopy was used to photograph the cells at 100X.

Live cells are distinguished by the presence of intracellular esterase; the enzyme converts non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein-green is well retained within live cells, producing an intense uniform green fluorescence. On the other hand, dead cells have damaged cell membrane that allows EthD-1 to enter the cell and undergo a 40-fold enhancement of fluorescence upon binding to nucleic acids, thus producing a bright red fluorescence in dead cells.

5.3.3 Statistical analysis

Each experiment was performed in triplicates or quadruplicates and repeated three times, data are represented as the mean \pm standard deviation. Statistical analysis was undertaken using Graphpad Prism 7.0. Two-way ANOVA was used to test significance, if significance was detected a Tukey's multiple comparisons test was conducted. Levels of significance are given in the text and considered statistically significant at a level of $P < 0.05$.

5.4 Results

5.4.1 Determination of cytotoxic effect of cobalt doped phosphate based glasses on normal uroepithelial cells

5.4.1.1 Indirect Contact

Figure 5.2 shows the viability of normal HUCs when exposed to media conditioned with various mol % of cobalt glasses. The pH of the conditioned media was recorded before and after conditioning time, and was within the optimum range. (Appendix Table A.1). The MTT assay was performed to assess the cytotoxic effects, cell viability was calculated as percentage of respective negative control.

As seen in Figure 5.2, the viability of HUCs was compromised with an increasing concentration of cobalt in media. Dissolution products of 10 mol % cobalt glass showed a significant cytotoxic effect against HUCs ($p < 0.0001$) when compared to negative control at 24, 48 and 72 hours. Similar effect was observed for dissolution products of 5 mol % cobalt glass, however the cytotoxic effect was less pronounced compared to 10 mol % cobalt glass dissolution products but significant compared to negative control ($p < 0.0001$). The least cytotoxic effect was seen when HUCs were treated with dissolution products of 3 mol % cobalt glass as the cell viability was 60%. Nonetheless, the result shows a significant ($p < 0.0001$) decrease in cell viability when treated for 24 hours, with further reduction in cell viability at 48 and 72 hours compared to the negative control.

Cytotoxic effect of un-doped phosphate glass was also studied along with cobalt doped phosphate glasses. It can be seen in figure 5.2, a reduction in cell viability was observed within 24 and 48 hours exposure to the dissolution products, 72.9% and 69.7% respectively ($p < 0.001$). However, a significant ($p < 0.0001$) decrease in cell viability was observed when the cells were treated for 72 hours with the dissolution products of un-doped phosphate glass.

The changes in the cell morphology following incubation with conditioned media were also observed using phase contrast microscopy. The cells maintained a cobbled shaped morphology for 24 and 48 hours for 3 and 5 mol % glass compositions, however appeared round when incubated 72 hours. Whereas, in 10 mol % glass cells appeared round after 24 hours (Figure A.7)

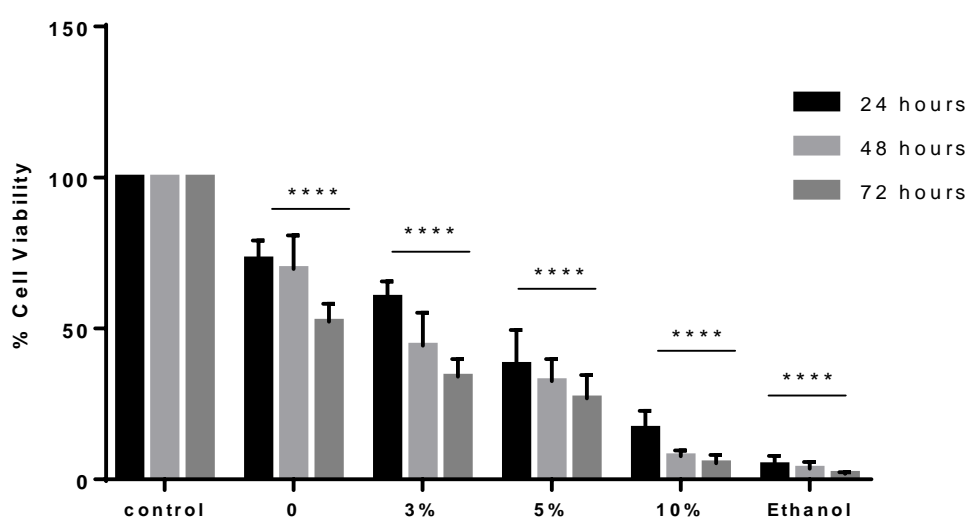


Figure 5.2: The effect of the dissolution products of cobalt doped phosphate based glasses (3%, 5% and 10 mol %) and un-doped phosphate glass on normal human uroepithelial cells. MTT assay was used to determine the cell viability following 24, 48 and 72 hours incubation with conditioned media. The data is represented as Mean \pm SD (N=3).

5.4.1.2 Evaluation of cell viability by Live/Dead staining

The cytotoxic effect of glass dissolution products was also assessed using Live/Dead staining, live cells are shown in green whereas dead cells in red. As seen in Figure 5.3, cells treated with dissolution products of 3 and 5 mol % cobalt glass for 24 hours showed similar viability as the cells cultured in urothelial cell media i.e. negative control. However, after 48 hours in culture, cells showed reduced viability compared to the 48 hours negative control and respective 24 hours dissolution products as a high number of cells were stained with EthD-1 (red - dead). HUCs treated with dissolution products of 10 mol % cobalt glass showed significant cytotoxic effects as none of the cells were stained with Calcein-AM (green – live), all cells lost viability within 24 hours of incubation with dissolution products.

Live/Dead staining of the un-doped phosphate glass showed that the dissolution products were non-toxic as similar proportion of live cells and very few dead cells were observed when compared to the cells grown in urothelial cell media. However, a decrease in live cells was observed when cells were exposed to the dissolution products for 48 hours. It was also observed that the live cells were clustered together.

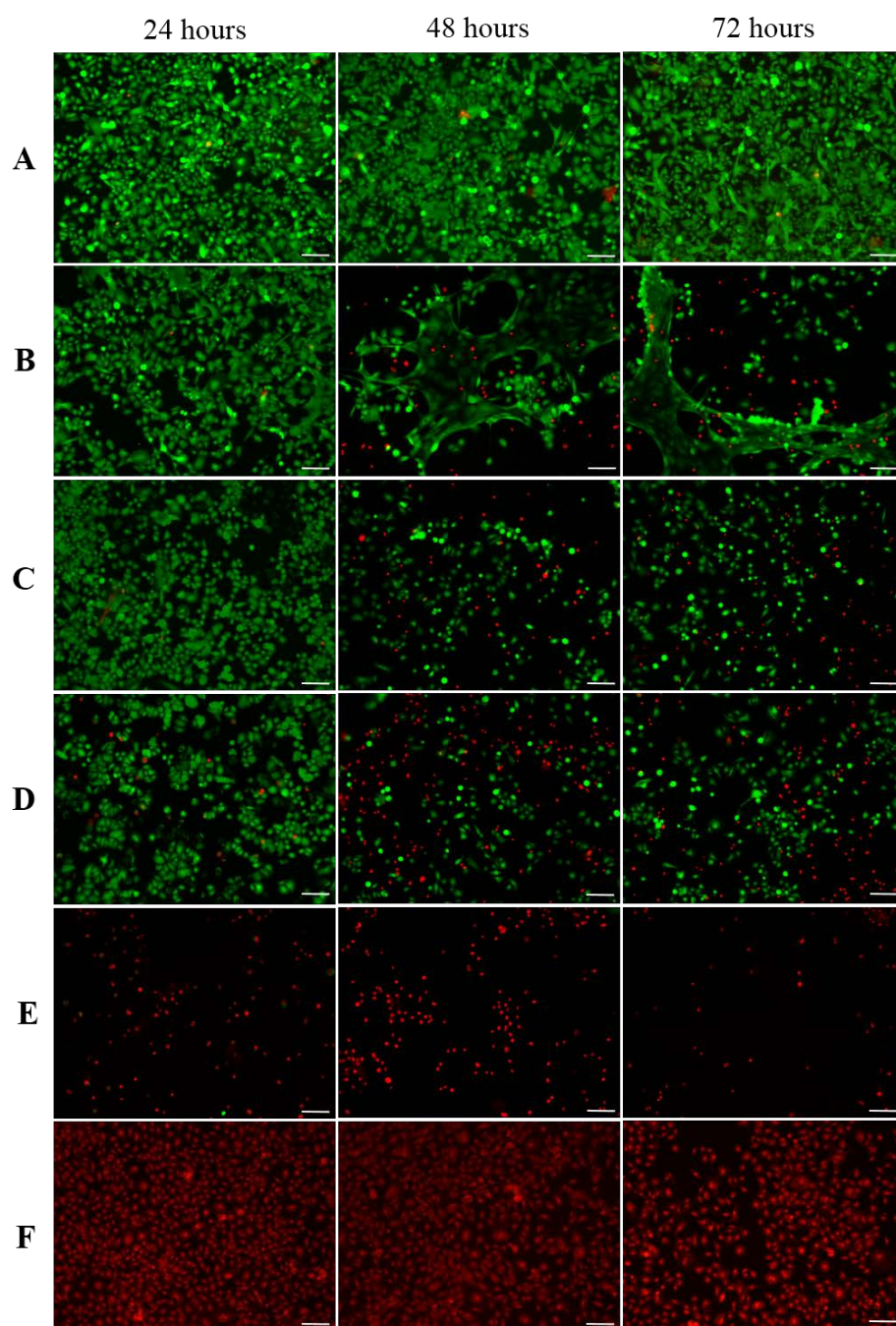


Figure 5.3: Fluorescence images of Live/dead staining of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of cobalt doped phosphate based glasses. Cells grown in (A) complete media, media conditioned with (B) un-doped phosphate glass, (C) 3% cobalt glass, (D) 5% cobalt glass (E) 10% cobalt glass and (F) cells treated with 70% (v/v) ethanol. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μm .

5.4.2 Determination of the cytotoxic effect of cobalt doped phosphate based glasses on T24 cells

5.4.2.1 Direct Contact

The cytotoxic effect of the glasses was determined by placing the glass discs on the cell monolayer. Following incubation for 24, 48 and 72 hours MTT assays were performed to evaluate the effect of glasses on the viability of T24 cells, which was calculated as the percentage of respective controls. As shown in Figure 5.4, the un-doped glass discs exhibited a non-significant decrease in the cell viability at all time points ($p > 0.9999$). According to the ISO-10993-5 guidelines no cytotoxic effect was observed with the cell viability of 94.9%, 88.4% and 77.8% at 24, 48 and 72 hours respectively. Similarly, for cobalt doped glasses a non-significant ($p > 0.9937$) decrease in cell viability was observed for 24 hours. The cell viability of 94.3%, 95.3% and 96.4% was observed for 3, 5 and 10 mol % glass discs.

The cell viability significantly reduced when cells were exposed to glass discs for 48 and 72 hours ($p < 0.0001$). At 48 hours, the cell viability reduced to 49.4% for 3 mol %, 49.0% for 5 mol % and 43.5% for 10 mol % cobalt glass, in comparison to the negative control. Moreover, at 72 hours the cytotoxic effect was even higher with cell viability of approximately 20% for all cobalt glass compositions. The changes in the cell morphology following incubation with glass discs were also observed using phase contrast microscopy. The cells maintained a cobbled shaped morphology for 24 and 48 hours, however appeared round when incubated with glass discs for 72 hours (Figure 5.5).

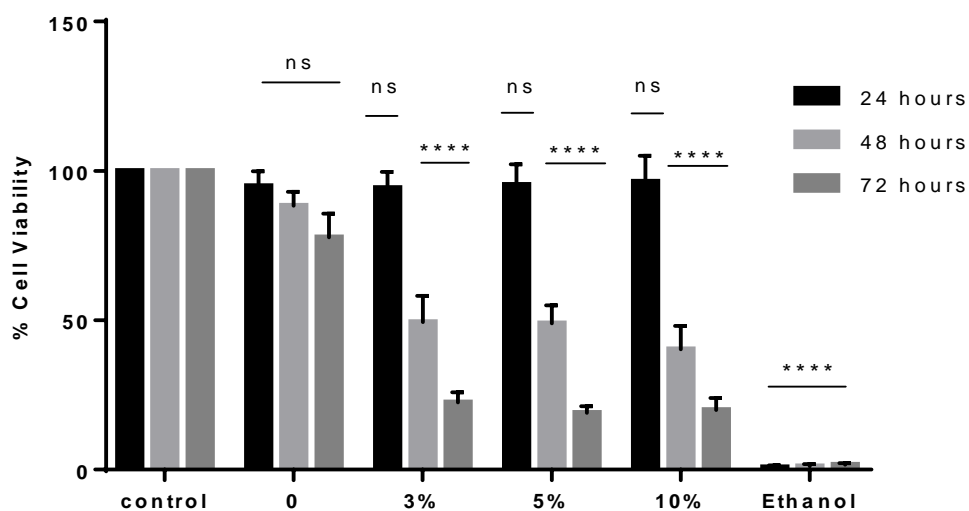


Figure 5.4: Cytotoxic evaluation, when in direct contact, of cobalt doped phosphate based glasses (3, 5 and 10 mol %) and un-doped phosphate glass on T24 cells. Glass discs were placed on cell monolayer and the MTT assay was used to determine the cell viability following 24, 48 and 72 hours incubation. The data is represented as Mean \pm SD (N=3).

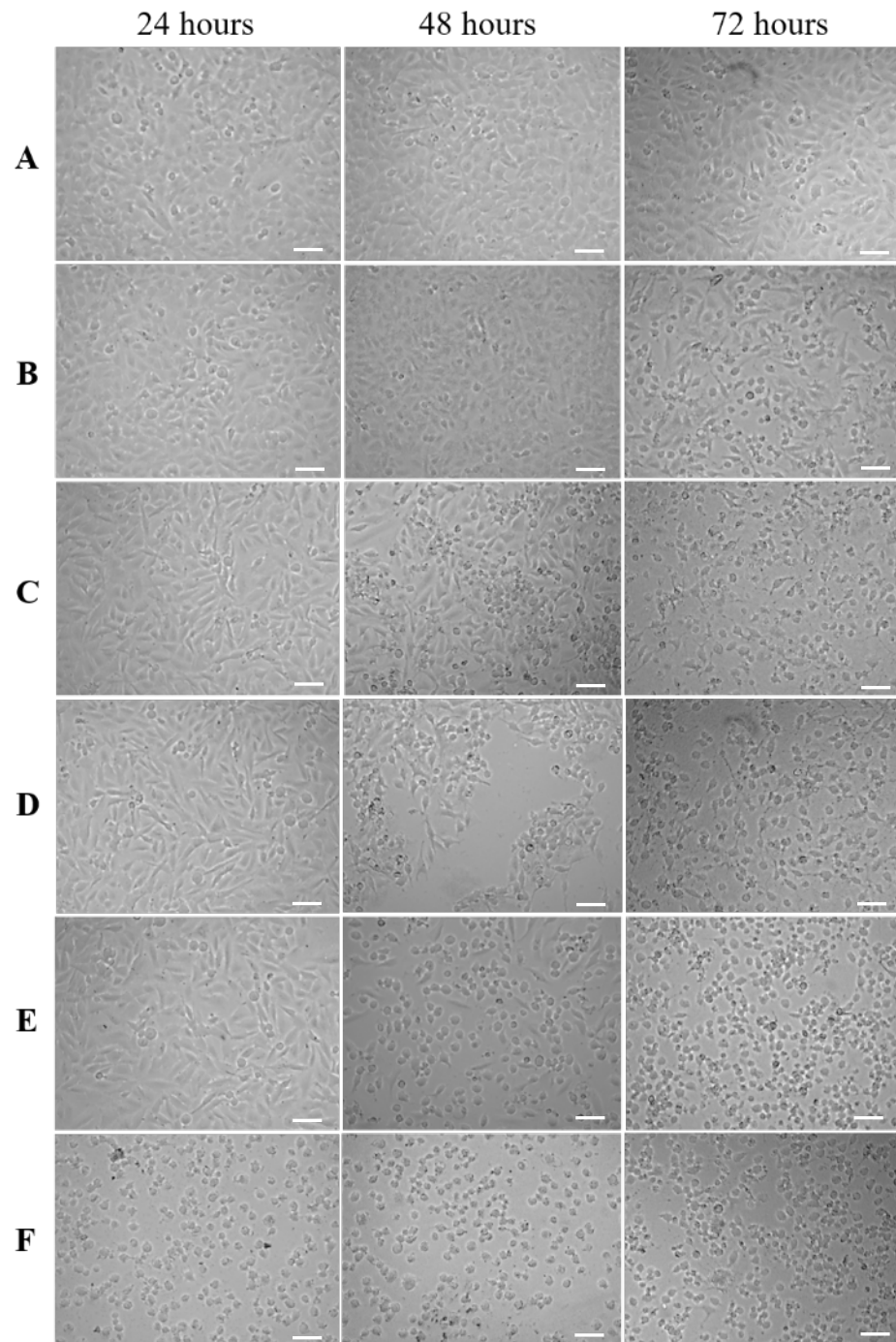


Figure 5.5: Phase contrast images of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of cobalt doped phosphate based glasses. Cells grown in (A) complete media, media conditioned with (B) un-doped phosphate glass, (C) 3% cobalt glass, (D) 5% cobalt glass (E) 10% cobalt glass and (F) cells treated with 70% (v/v) ethanol. Scale bar indicates 100 μm .

5.4.2.2 Indirect contact

Cytotoxic effect was determined both with and without buffering the pH of the conditioned media. pH readings of the media before and after buffering are given in Table A.2. The cell viability after treating the cells with conditioned media, for 24, 48 and 72 hours, was quantified using MTT assay.

According to the international standard protocol for testing medical devices for cytotoxicity, if the cell viability is less than 70% compared to the negative control then the test material is considered cytotoxic. As seen in Figure 5.6A, the cell viability significantly reduced for most of the glasses at each time point except for 3% and 5% glass compositions at 24 hour. The cell viability for 3% glass reduced to approximately half within 24 hours ($p < 0.0001$) and further reduction was observed for 48 and 72 hours ($< 15\%$), with statistical significance of $p < 0.0001$. Similar data was obtained for 5% glass; however, the highest effect was seen for 10% glass where percentage cell viability was less than 5 even after 24 hours, significantly reduced in comparison to control media ($p < 0.0001$). Since human body has evolved mechanisms to buffer pH changes and maintain it within optimum range therefore pH of the conditioned media was buffered to represent *in vivo* conditions.

The pH buffered analysis was undertaken to rule out the effect of pH on the cell viability, if any. The cell viability improved for 3 mol % glass composition at 24 hours as the reduction in viability was non-significant. However, at 48 and 72 hours significant decrease in cell viability was observed ($p < 0.001$). Between 24 hours and 48 hours, the percentage cell viability increased from 50 to 85 suggesting lack of cytotoxicity of 3% cobalt glass. On the other hand, even though the cell viability improved for 5 mol % glass composition however it remained below 70% hence cytotoxic. Contrary to 3 and 5 mol % glasses no effect of pH buffering was observed for 10 mol % cobalt glass (Figure 5. 6B) which suggests toxicity due to cobalt ions rather than the pH.

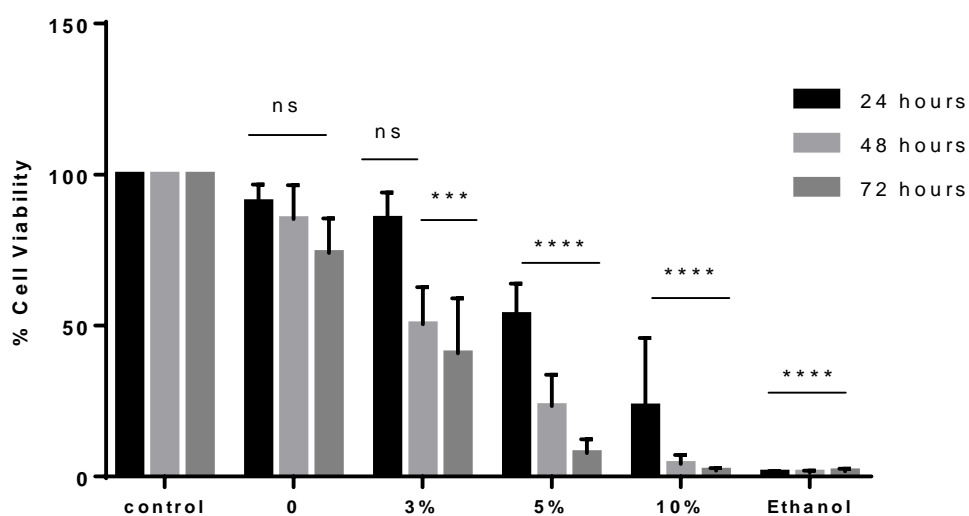
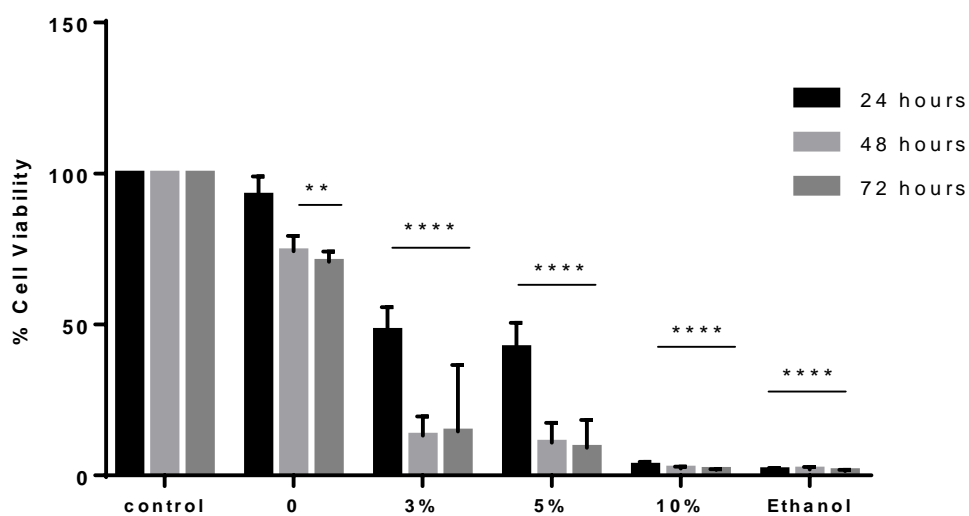


Figure 5.6: The effect of the dissolution products of cobalt doped phosphate based glasses (3%, 5% and 10 mol %) and un-doped phosphate glass on T24 cells. MTT assay was used to determine T24 cell viability following 24, 48 and 72 hours incubation with conditioned media without pH buffering (A) and with pH buffering (B). The data is represented as Mean \pm SD (N=3).

5.4.2.3 Evaluation of cell viability by Live/Dead staining

Figure 5.7 and 5.8 show the fluorescent microscopy of T24 cells following incubation with dissolution products of cobalt doped glasses along with controls for 24, 48 and 72 hours stained with Calcein-AM and Ethidium homodimer-1. Micrographs are representative of two replicates. The data obtained corresponded the results from indirect contact experiment. As it can be seen in the Figure 5.7 and 5.8, cells in negative control (A) and un-doped glass conditioned media (B) were stained green (live) whereas very few were stained red (dead). On the other hand, when pH buffered and non-buffered results were compared, the viability was seen to improve for 3 and 5 mol % buffered conditioned media, as the number of live cells increased (Figure 5.8) compared to non-buffered conditioned media (Figure 5.7). However, as seen from Figure 5.7 and 5.8, no live cells were seen for 10 mol % cobalt glass composition, even when the pH was buffered which is indicative of high toxicity of the glass due to metal ions.

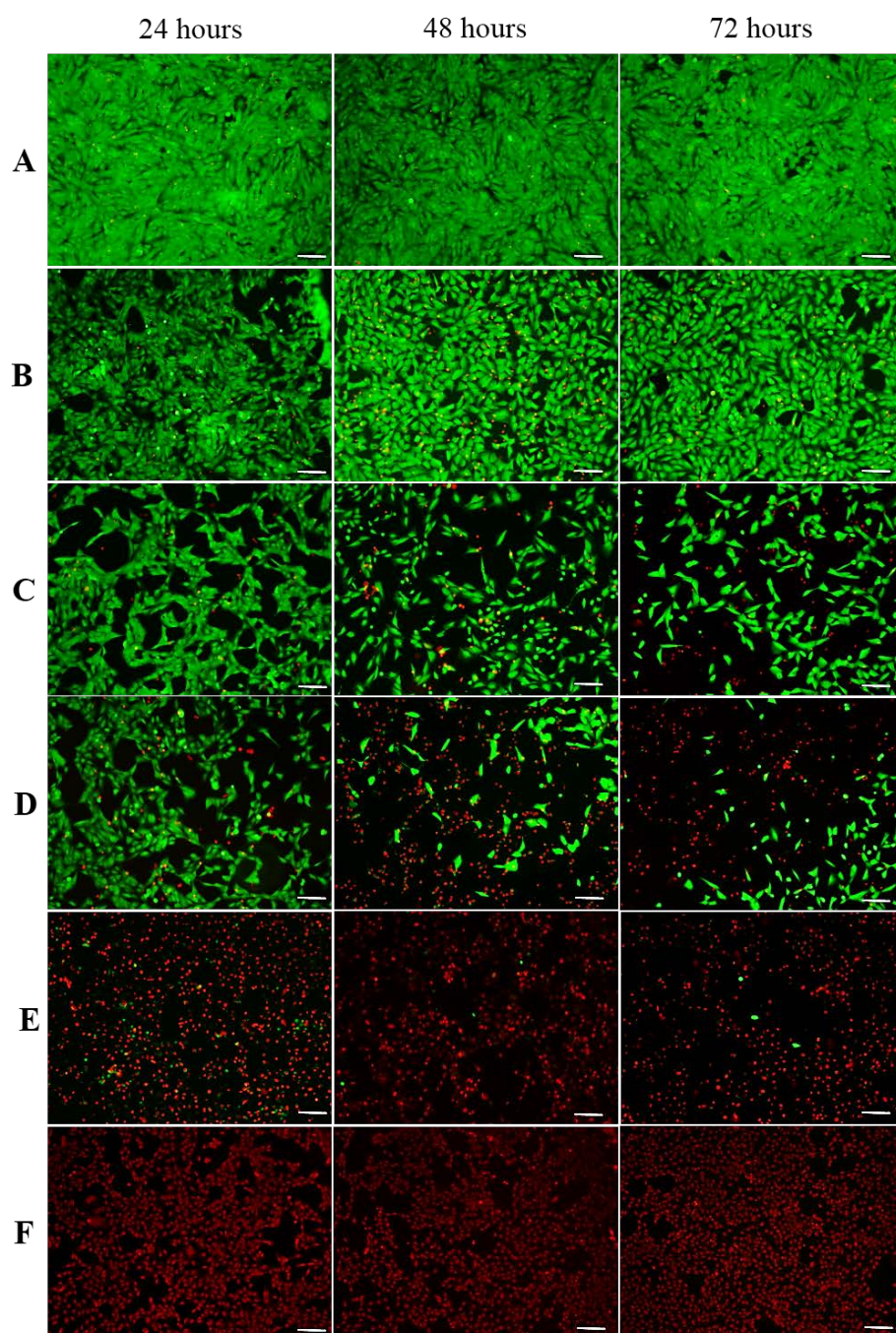


Figure 5.7: Live/dead staining of T24 cells cultured with media conditioned with cobalt doped phosphate based glasses at 24, 48 and 72 hours; without pH buffering. Cells grown in (A) complete media, media conditioned with (B) undoped phosphate glass, (C) 3% cobalt glass, (D) 5% cobalt glass (E) 10% cobalt glass and (F) cells treated with 70% v/v ethanol, at time point 24, 48 and 72 hours. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μm .

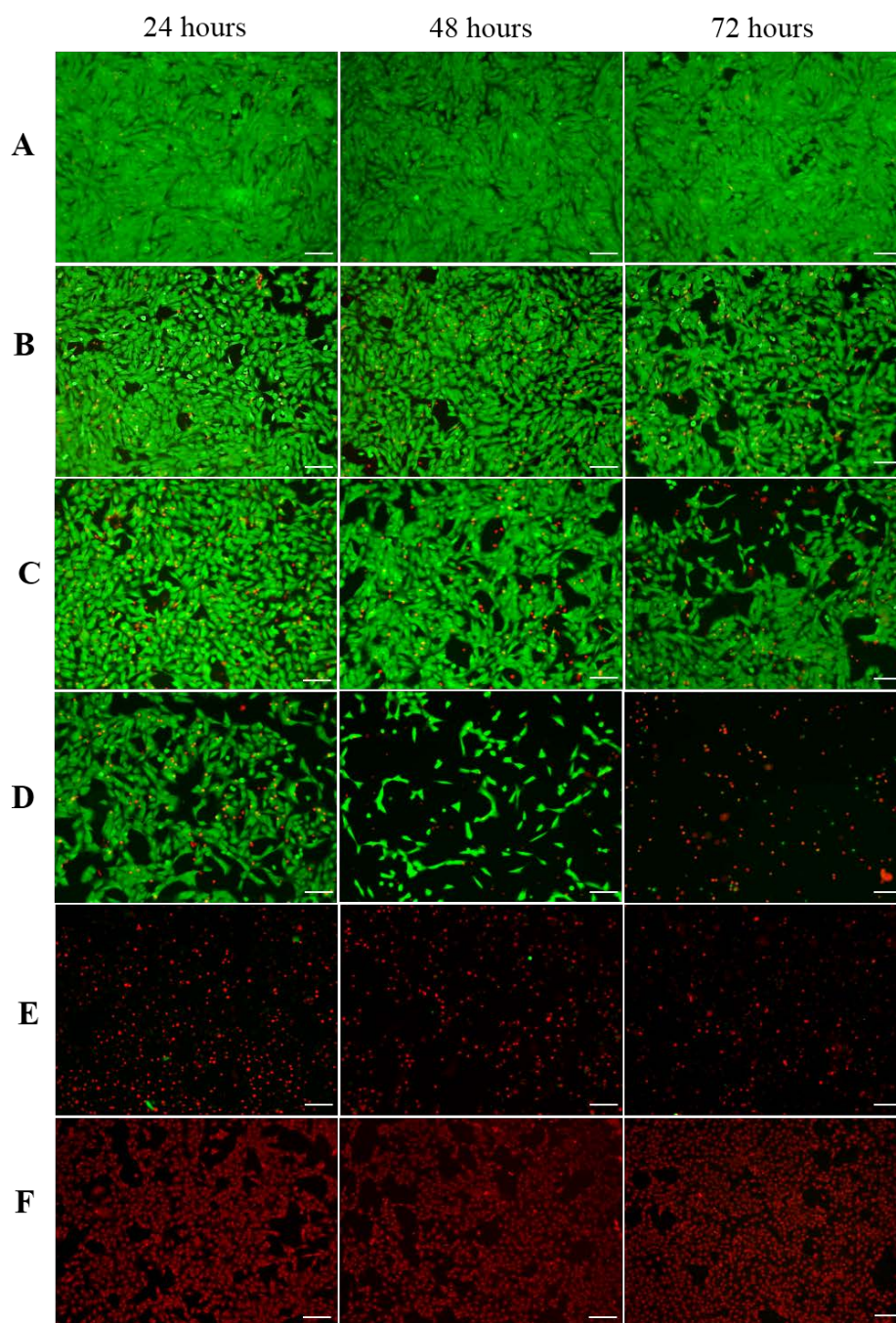


Figure 5.8: Live/dead staining of T24 cells cultured with media conditioned with cobalt doped phosphate based glasses at 24, 48 and 72 hours; with pH buffering. Cells grown in **(A)** complete media, media conditioned with **(B)** un-doped phosphate glass, **(C)** 3% cobalt glass, **(D)** 5% cobalt glass **(E)** 10% cobalt glass and **(F)** cells treated with 70% v/v ethanol, at time point 24, 48 and 72 hours. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μ m.

5.4.3 Determination of the cytotoxic effect of zinc doped phosphate based glasses on normal uroepithelial cells

5.4.3.1 Indirect Contact

The effect of dissolution products of zinc doped phosphate based glasses on normal human uroepithelial cells was evaluated. pH measurements of the media and media with dissolution products were determined prior to and after conditioning time, both readings were within the physiological pH range acceptable for cell growth and maintenance (Table A.1). Figure 5.9 shows the viability of normal HUCs when exposed dissolution products of various mol % of zinc glasses. MTT assay was performed to assess the cytotoxic effects, cell viability was calculated as percentage of respective negative control.

The viability of HUCs was compromised when treated with dissolution products of zinc doped glasses compared to negative control as well as the un-doped phosphate based glass. A significant decrease ($p < 0.0001$) in the cell viability was seen when treated with dissolution products of 3 mol % zinc doped glass. Cell viability reduced to 24.0%, 19.8% and 19.8% at 24, 48 and 72 hours respectively. Furthermore, dissolution products of 5 and 10 mol % zinc doped glasses exerted a greater cytotoxic effect with cell viability of less than 5% at 24, 48 and 72 hours ($p < 0.0001$) compared to respective negative control. On the other hand, dissolution products from un-doped phosphate glass exhibited a reduction in cell viability within 24 and 48 hours exposure to the dissolution products, 72.9% and 69.7% respectively ($p < 0.001$). However, a significant ($p < 0.0001$) decrease in cell viability was observed when the cells were treated for 72 hours with the dissolution products of un-doped phosphate glass.

Changes in cell morphology following exposure to the dissolution products were also observed under a light microscope. As the concentration of dissolution products increased in media, the typical cobblestone shape of the HUCs changed into a round shape. A large number of the cells detached from the surface of the well i.e. lost viability (Figure A.9).

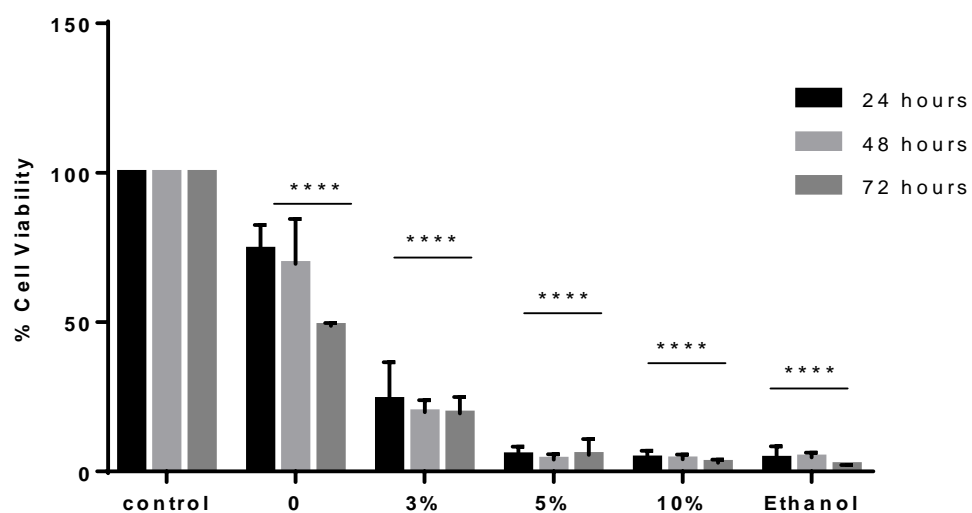


Figure 5.9: The effect of the dissolution products of zinc doped phosphate based glasses (3%, 5% and 10 mol %) and un-doped phosphate glass on normal human uroepithelial cells. MTT assay was used to determine the cell viability following 24, 48 and 72 hours incubation with conditioned media. The data is represented as Mean \pm SD (N=3).

5.4.3.2 Evaluation of cell viability by Live/dead staining

Live/Dead staining of HUCs was also performed to assess the cytotoxic effects of glass dissolution products. Figure 5.10 shows fluorescent images of HUCs where live cells are shown in green and dead cells in red. The un-doped phosphate glass showed that the dissolution products were non-toxic as similar proportion of live cells and very few dead cells were observed when compared to the cells grown in urothelial cell media. However, a slight decrease in live cells was observed when cells were exposed to the dissolution products for 48 hours. Whilst no or very few dead cells were observed for un-doped glass dissolution products, a considerable cytotoxic effect was observed when cells were cultured with dissolution products of zinc doped glasses as no live (green) cells were seen at any time point studied compared to negative control.

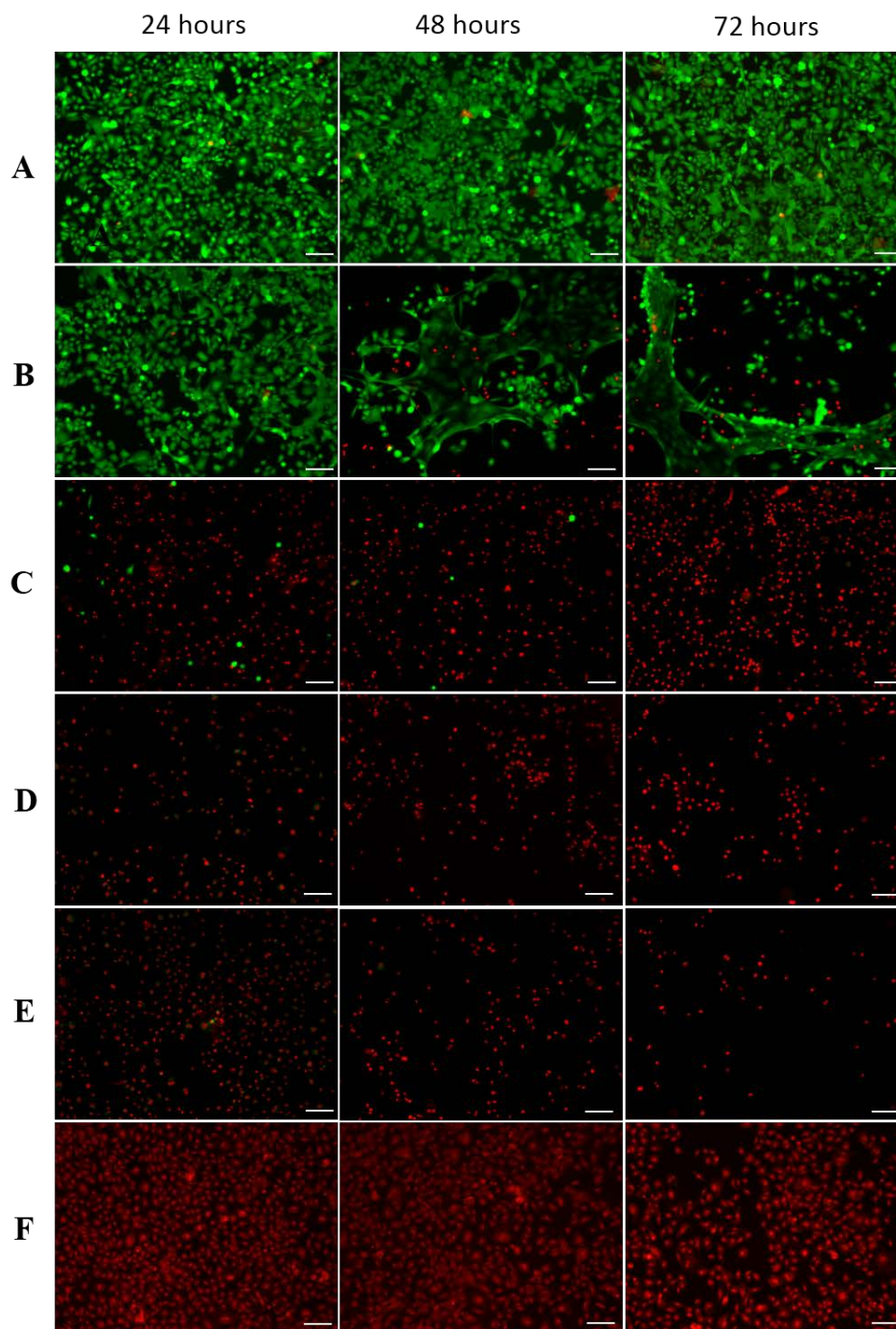


Figure 5.10: Fluorescence images of Live/dead staining of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of zinc doped phosphate based glasses. Cells grown in (A) complete media, media conditioned with (B) un-doped phosphate glass, (C) 3% zinc glass, (D) 5% zinc glass (E) 10% zinc glass and (F) cells treated with 70% (v/v) ethanol. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μm .

5.4.4 Determination of the cytotoxic effect of zinc doped phosphate based glasses on T24 cells

5.4.4.1 Direct Contact

The effect of placing glass discs on the surface of cell monolayer was assessed. This was achieved by performing MTT assay after exposing cells to glass discs for 24, 48 and 72 hours and the cell viability was calculated as the percentage of respective controls. The changes in cell morphology was also determined under light microscope.

Cell viability significantly ($p < 0.0001$) decreased when cell monolayers were exposed to zinc doped glasses. Figure 5.11 shows that 3, 5 and 10 mol % zinc doped glasses had pronounced cytotoxic effect with viability reduced to 12.9%, 10.5% and 2.2% respectively within 24 hours. Cell viability was less than 2% at 48 and 72 hours for all glass compositions. This was also seen in phase contrast microscopy of the cells when exposed to glass discs. Cells lost their characteristic shape within 24 hours, appeared round and detached themselves from the surface of the well (Figure 5.12). On the other hand, the un-doped glass discs exhibited a non-significant ($p > 0.9907$) decrease in cell viability at all time point (94.9%, 88.4% and 77.8% at 24, 48 and 72 hours respectively).

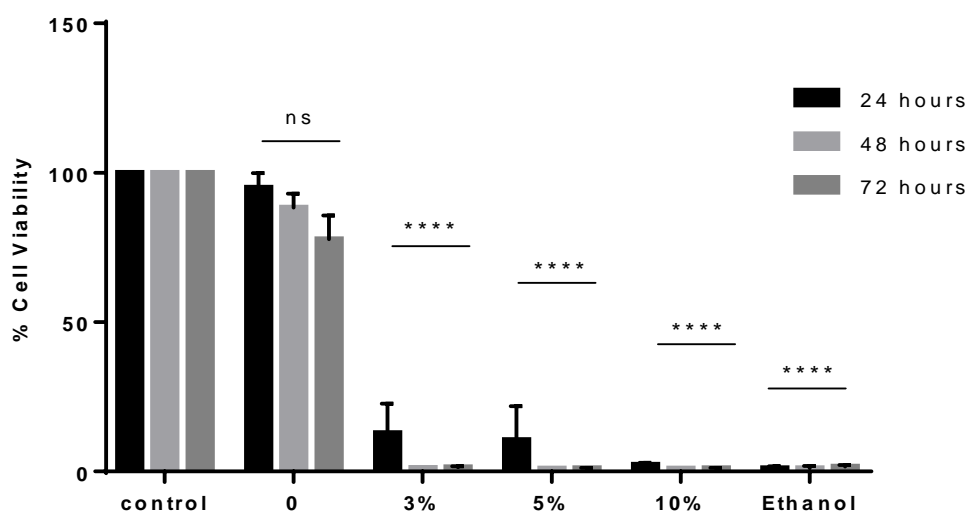


Figure 5.11: Cytotoxic evaluation, when in direct contact, of zinc doped phosphate based glasses (3, 5 and 10 mol %) and un-doped phosphate glass on T24 cells. Glass discs were placed on cell monolayer and MTT assay was used to determine the cell viability following 24, 48 and 72 hours incubation. The data is represented as Mean \pm SD (N=3).

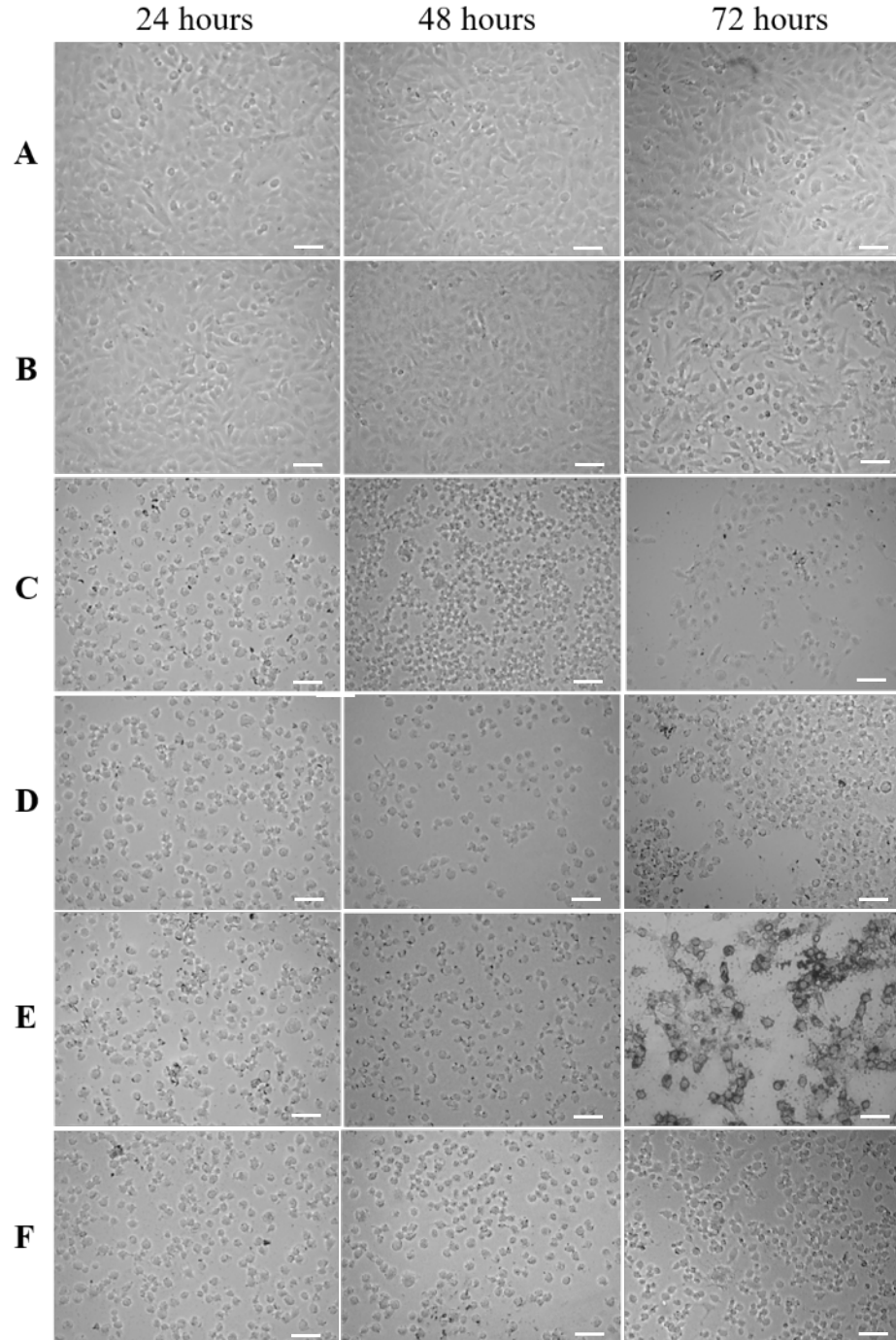


Figure 5.12: Phase contrast images of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of zinc doped phosphate based glasses. Cells grown in (A) complete media, media conditioned with (B) un-doped phosphate glass, (C) 3% zinc glass, (D) 5% zinc glass (E) 10% zinc glass and (F) cells treated with 70% (v/v) ethanol. Images were taken at 100x magnification. Scale bar indicates 100 μm .

5.4.4.2 Indirect contact

The cytotoxic effect was determined both; with and without buffering the pH of the conditioned media. pH readings of the media before and after buffering are given in Table A.2. The cell viability after treating the cells with conditioned media, for 24, 48 and 72 hours, was quantified using MTT assay.

Figure 5.13 shows non-cytotoxic effects of dissolution products of zinc doped phosphate glasses on viability of T24 cells. A non-significant decrease in the cell viability was seen for 3, 5 and 10 mol % zinc glasses compared to negative control as well as the un-doped phosphate based glass. For 3 mol % glass dissolution products the viability was 93.5%, 96.6% and 88.9% at 24, 48 and 72 hours respectively, which was comparable even after pH buffering of the conditioned media.

For 5 and 10 mol % zinc glasses a slight cytotoxic effect was observed with viability of 87.2%, 86.5% and 73.5% for 5 mol %; 79.5%, 71.6% and 66.7% for 10 mol % zinc doped glass dissolution products. However, unlike 3 mol % zinc glass products the viability improved following pH neutralisation of the conditioned media; 94.2%, 88.2% and 85.0% for 5 mol %; 95.9%, 81.4% and 67.6% for 10 mol % zinc doped glass dissolution products.

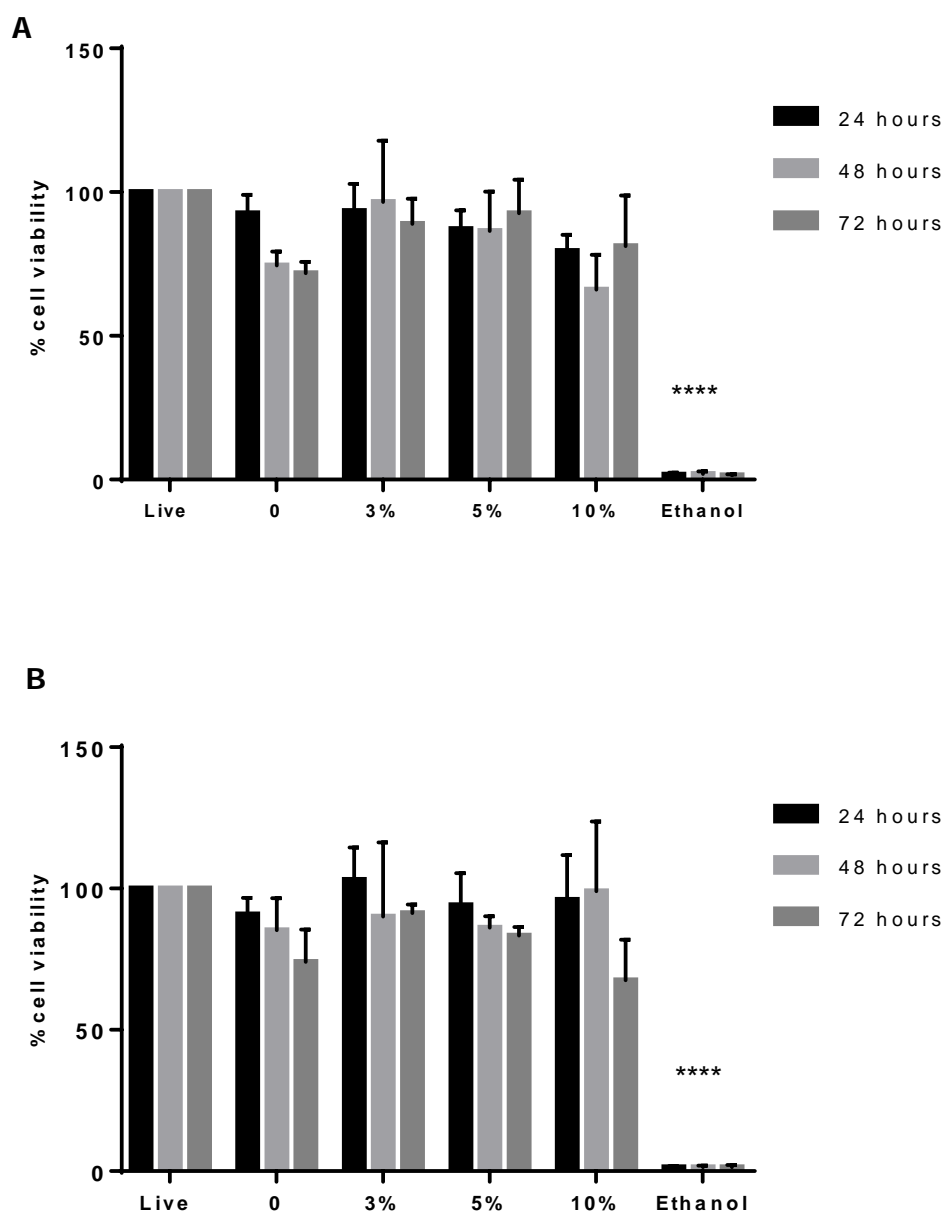


Figure 5.13: The effect of the dissolution products of zinc doped phosphate based glasses (3%, 5% and 10 mol %) and un-doped phosphate glass on T24 cells. MTT assay was used to determine T24 cell viability following 24, 48 and 72 hours incubation with conditioned media without pH buffering (**A**) and with pH buffering (**B**). The data is represented as Mean \pm SD (N=3).

5.4.4.3 Evaluation of cell viability by Live/dead staining

The cytotoxic effects of dissolution products were also assessed by Live/Dead staining of the cells with Calcein-AM green and EthD-1. The effect of dissolution products was evaluated on cell viability with and without pH buffering of the conditioned media. As seen in Figure 5.14, 3 mol % zinc glass dissolution products, a large number of cells were stained with Calcein-AM (green – live) and a very few or none with EthD-1 (red – dead), when compared with respective negative control and un-doped glass. However, quite a few cells were stained with EthD-1 in 5 and 10 mol % zinc doped glasses. When compared to buffered dissolution products (Figure 5.15) the cell viability appeared to improve for 5 and 10 mol % zinc glass dissolution products, as none of the cells were stained with EthD-1. Overall, by looking at the confluency of the culture in Figure 5.15 shows that the pH buffered conditioned media appeared to enhance the cell growth.

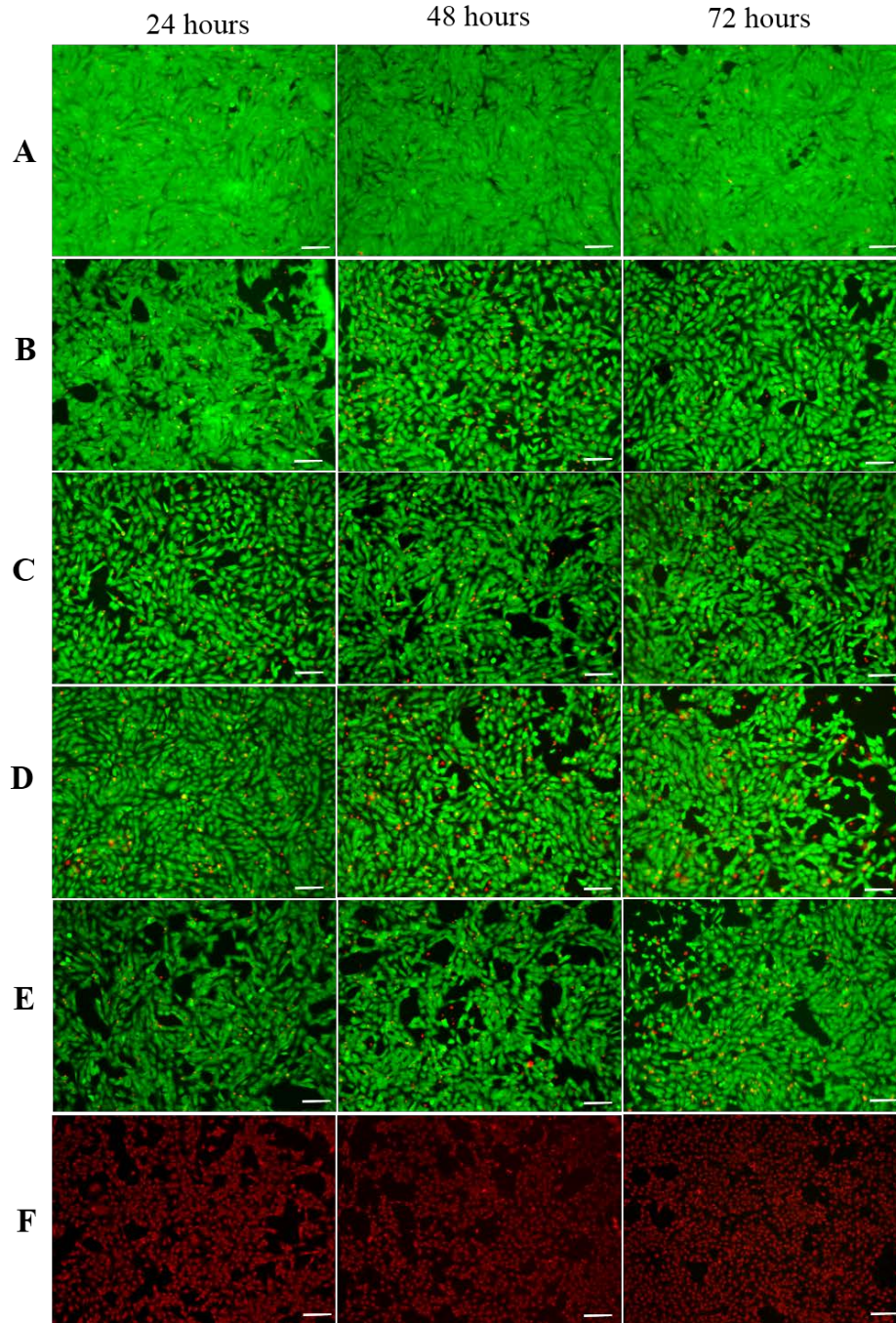


Figure 5.14: Live/Dead staining of T24 cells cultured with media conditioned with zinc doped phosphate based glasses at 24, 48 and 72 hours; without pH buffering. Cells grown in (A) complete media, media conditioned with (B) undoped phosphate glass, (C) 3% zinc glass, (D) 5% zinc glass (E) 10% zinc glass and (F) cells treated with 70% (v/v) ethanol at time point 24, 48 and 72 hours. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μ m.

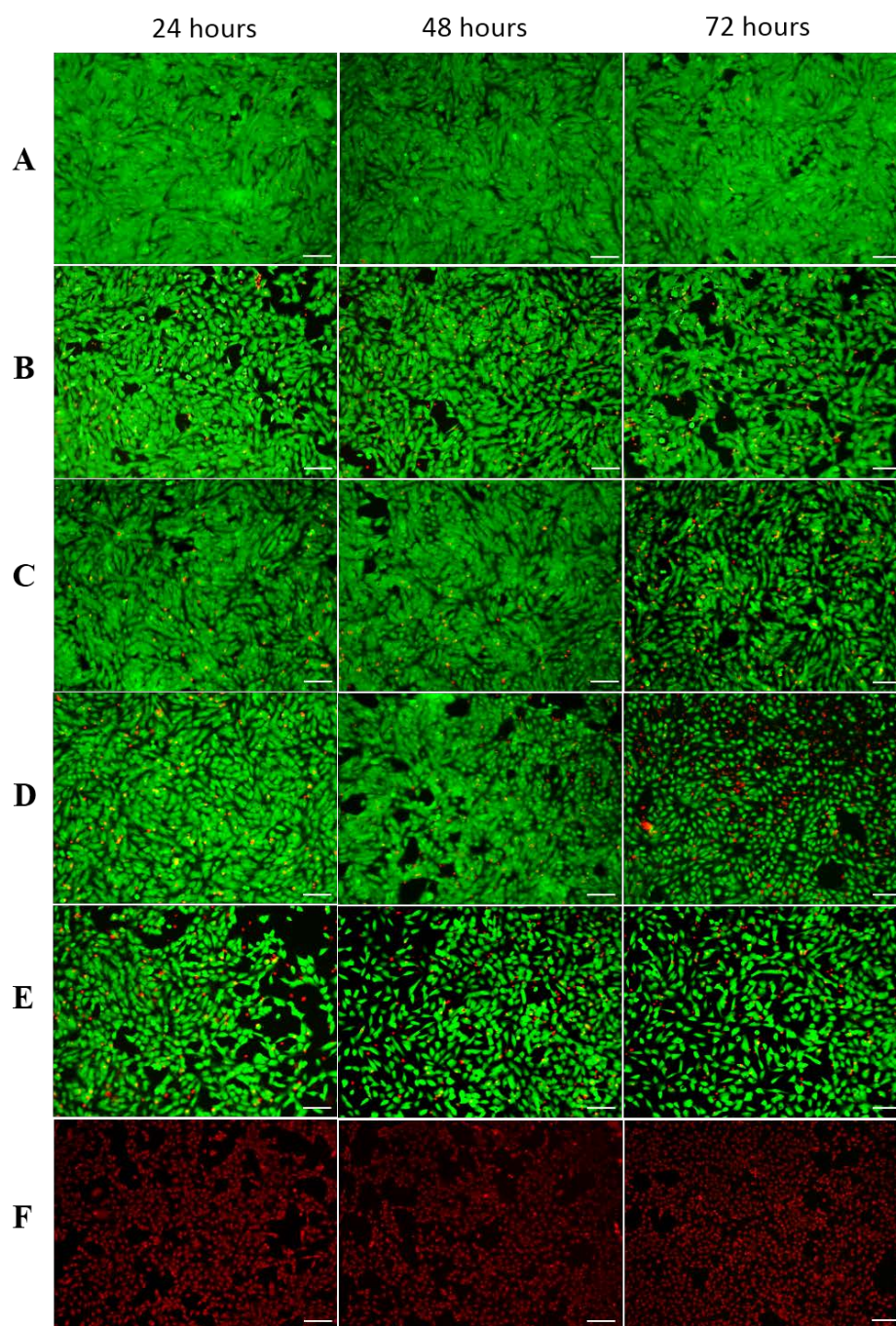


Figure 5.15: Live/dead staining of T24 cells cultured with media conditioned with zinc doped phosphate based glasses at 24, 48 and 72 hours; with pH buffering. Cells grown in **(A)** complete media, media conditioned with **(B)** un-doped phosphate glass, **(C)** 3% zinc glass, **(D)** 5% zinc glass **(E)** 10% zinc glass and **(F)** cells treated with 70% (v/v) ethanol at time point 24, 48 and 72 hours. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100 μ m.

5.5 Discussion

Phosphate glasses have been extensively investigated for their biocompatibility as discussed in section 5.1. The aim of the study was to investigate the cytotoxic effects of 3, 5 and 10 mol % cobalt doped and zinc doped phosphate glasses on the growth and viability of normal HUCs and T24 cells. This was achieved by assessing the effect of dissolution products of the glasses and by placing the glass discs directly on cell monolayers i.e. direct contact test. The dissolution products of cobalt doped glasses were found to exhibit a strong cytotoxic effect against normal HUCs, a concentration dependent decrease in cell viability was observed which was also shown by Live/Dead staining of the cells (Figure 5.2 and 5.3). Whilst the pH of glass dissolution products remained within optimum range in UCM, it was slightly higher than the optimum range in McCoy's 5A medium, hence pH buffered and un-buffered studies were performed with T24 cells. Overall, viability of T24 cells was compromised in a dose dependent manner in both pH buffered and un-buffered analysis. Thus, a reduced cell viability of both cell types was demonstrated with dissolution products of cobalt doped glasses where 10 mol % glass was the most toxic composition. The data obtained was supported by the fluorescent and phase contrast microscopy.

The decrease in cell viability could be attributed to the ionic strength of the conditioned media. Many studies have demonstrated that the cytotoxicity occurs due to glass degradation and associated change in the ionic strength as well as the pH of the solution. Bioactive glasses when immersed in an aqueous media degrade and release ionic products, such as phosphorus, calcium and sodium in the present case, which if higher than the optimum range are detrimental to cells. The human body requires these elements to perform various functions. For instance, calcium is required for membrane permeability, muscle contraction whereas phosphorus plays a role in the synthesis of phospholipids and phosphoproteins. Similarly, sodium is present in plasma and help regulate nerve impulse and maintain fluid balance in the body [24].

Epithelial cells, like all other cells, have several mechanisms to tightly regulate the calcium levels, according to Joost *et al.*, [25], optimum calcium tolerance level of epithelial cells is approximately 2.5mM/100ppm, similarly sodium in plasma is 3200 ppm [26]. A study conducted by Franchini *et al.*, [27] on gallium phosphate glasses reported 101 ppm and 3490 ppm concentration of calcium and sodium ions respectively, in 24 hours, as non-toxic. Additionally, 88-100 ppm of calcium ions is reported as the concentration needed for the up regulation of several osteogenic genes [7, 28, 29]. In the present analysis, the most soluble glass i.e. un-doped phosphate glass (P_2O_5 -CaO- Na_2O) released 64 ppm of calcium and 50 ppm of sodium within 24 hours of immersing in distilled water, therefore the cell toxicity observed in the study is unlikely due to calcium or sodium ions. Additionally, *in vitro* cytotoxic evaluation of 50 mol % phosphate based glasses have shown the non-toxic effects of glasses [8]. The non-toxic nature of the un-doped glass (P_2O_5 -CaO- Na_2O) has also been shown here as the un-doped glass shows proliferative effect on cell viability when compared to doped glasses (Figure 5.2 - 5.14). Therefore, the decrease in cell viability reported here is likely due to the cobalt ions.

Whilst biocompatibility of glasses has been extensively studied, there is limited literature available on cobalt doped bioactive glasses. The available work covers the angiogenic potential of cobalt doped silicate glasses in bone tissue engineering [30]. While no data is available on cytotoxic effect of cobalt doped phosphate glasses on epithelial cells, some of the studies have stated the possible optimum range of the cobalt ions. Hoppe *et al.*, has reported 12, 24 and 48 ppm of cobalt ion as therapeutically active range that stimulated migration, promoted proliferation and tubule like structure formation of umbilical cord blood derived CD133(+) cells [31]. In another study 12 ppm $CoCl_2$ was shown to upregulate endothelin-1 gene in human microvascular endothelial cells [32]. Similarly, Lucas *et al.*, found that cobalt ions < 10 $\mu g/ml$ (10 ppm) were well tolerated by osteoblast as the effect of the ions on cell proliferation and function was non-significant. However, cobalt ions > 10 ppm has shown cytotoxic effects on osteoblast like MG63 cells [33] and bone marrow derived stem cells [30]. In the present study,

the ionic concentration of cobalt ion was less than 10 ppm which per the above studies is within therapeutic range. Nevertheless, a significant cytotoxic effect of the cobalt ions was seen on uroepithelial cells studied, even though the ionic concentration was within an acceptable range. The cytotoxic effect of cobalt exhibited in this study could be attributed to the fact that various cells are likely to have different tolerance level for ions. To date no studies have been conducted on cytotoxicity of cobalt ions on uroepithelial cells. Therefore, this study is the first to evaluate and report cytotoxic effect of cobalt ions on human uroepithelial cells.

It is evident from the data that the un-doped glass dissolution products have non-significant effect on cells compared to cobalt doped samples. High cobalt doses have been shown to induce cell death via a variety of mechanisms. Cobalt ions act as antagonist for calcium channels therefore inhibit the calcium signalling pathways [34]. Once inside the cell, cobalt ions can inhibit crucial enzymes due to their high affinity for sulphhydryl groups and hinder metabolic processes [35, 36]. Cobalt ions also induce oxidative stress by producing reactive oxygen species that cause DNA, proteins and lipid damage [34, 37]. Lucas *et al.*, reported that the DNA synthesis in human osteoblasts like cells was inhibited by cobalt ions in a dose dependent manner [38]. Similarly, cobalt has been shown to inhibit enzymes involved in DNA repair and synthesis thus leading to chromosome aberrations [39, 40]. Xie *et al.*, [41] reported chromosomal aberrations in normal primary human lung epithelial cells due to soluble and particulate cobalt. Most importantly cobalt ions induce hypoxia (low oxygen pressure) and severe and prolonged hypoxia initiates cell death by apoptosis [37, 42].

Whilst high cobalt concentrations have been shown to be cytotoxic in various studies, several other studies have shown the controlled release of cobalt ions can promote cell growth by inducing hypoxia. Certain metal ions such as cobalt, copper, nickel and vanadium have been shown to induce oxidative stress by mimicking hypoxia conditions [43]. Whilst severe and

prolonged hypoxia has been shown to induce cell death *in vitro*, however under acute hypoxic conditions cells may adapt to the stress by initiating a survival mechanism. One of the mechanism is increased vascularization, by expressing genes that are related to angiogenesis. *In vivo* hypoxia stabilizes hypoxia inducible factor 1 alpha (HIF-1 α) and stimulate expression of Vascular Endothelial Growth Factor (VEGF) and erythropoietin (EPO) genes, which subsequently activate the cascade affecting vascularization and angiogenesis [30, 43]. Therefore, it is difficult to translate the *in vitro* cytotoxic effect of cobalt to *in vivo* conditions.

Many studies have reported the cytotoxic effect of bioactive glasses is due to the change in pH as the glasses degrade. The pH of the conditioned media for HUCs was neutral however the pH of the conditioned media for T24 cells was neutralised to investigate if the cell viability is influenced by the pH of the conditioned media. Cell viability of T24, following pH neutralisation, was similar for all compositions except as a dose dependent decrease in viability was observed except for 3 mol % cobalt doped glass. It was found that the cell viability improved following pH neutralisation by 40%, 37% and 27% at 24, 48 and 72 hours respectively (Figure 5.6). The reduced cell viability observed in 3 mol % glasses is an *in vitro* phenomenon and the viability following pH neutralisation is more representative of *in vivo* conditions as human body has naturally buffering system where pH is maintained within an optimum range, approximately 7.0. Nevertheless, these findings suggest that the cytotoxic effect observed against HUCs and T24 cells is due to the presence of dissolution products of cobalt glass.

Whilst dissolution products of cobalt doped phosphate glasses showed a concentration dependent effect on cell viability, dissolution products of zinc doped phosphate glasses showed a significant decrease in HUCs viability at all concentrations (Figure 5.9). Zinc has been shown to play a key role in various biological process such as development of skeletal system, DNA replication and protein synthesis [44-46]. However, higher concentration of zinc has detrimental effects of cells.

Aina *et al.*, [47] showed that zinc concentrations in the range of 2-8 ppm are cytotoxic to human osteoblasts. Similar results were seen against endothelial cells where zinc concentration of 2.7 ppm caused cell death, whereas 1.1 ppm showed cell proliferation [48]. In the present study, the glass composition containing the lowest mol % of zinc oxide (3 mol %) had approximately release rate of 9 ppm which per above studies is cytotoxic.

Whilst cytotoxic effect was observed against primary HUCs, a proliferative effect on viability of transitional carcinoma T24 was observed both with and without buffering the pH of the dissolution products (Figure 5.13). Zinc has been shown to selectively kill cancer cells due to high susceptibility of cancer cells to ROS. A study conducted on ZnO nanoparticles showed cell death in breast and prostate cancer cells while no major effect was observed in the respective normal cells [49]. The lack of cytotoxic effect observed in the present study against T24 cells could be attributed to the fact that different cell types have different tolerance for a given ion. Additionally, the two cell types were cultured in different media and each medium has a diverse range of components containing ions such as Cl^- , HCO_3^- , CO_3^{2-} and proteins which makes it difficult to maintain the ionic form of zinc. It is likely that the zinc ions in McCoy's 5A media form soluble or insoluble complexes thus depleting the media of zinc ions and thus no cytotoxic effect was observed.

The effect of direct contact of cobalt doped phosphate based glasses with cell monolayer showed a non-significant effect on cell viability for 24 hours, whereas a significant reduction ($p < 0.001$) in cell viability was observed at time point 48 and 72 hours (Figure 5.4). On the other hand, T24 viability significantly reduced when the cells were exposed to zinc doped phosphate based glasses directly. The reduction in cell viability in direct contact experiment is likely due to the drastic change in the pH of the cell culture media when cell monolayer was exposed to glass discs. Several studies have reported that the decrease in cell viability following exposure to bioactive

glasses is due to the associated pH changes. The experiment was conducted in 6 wells plate where the volume of the media was two millilitres compared to indirect contact experiments where 25 ml media was used. Small volume of cell culture media is unlikely to buffer the pH changes, therefore high pH reduces cell growth and proliferation.

5.6 Conclusion

The dissolution products of 3, 5 and 10 mol % cobalt or zinc doped phosphate based glasses showed a significant cytotoxic effect on primary human uroepithelial cells. However, a proliferative effect of dissolution products of zinc doped glasses was observed against T24 cells, whereas dissolution products of cobalt doped glasses demonstrated cytotoxic effect even when the pH was neutralised. The results from direct contact experiments show that cobalt or zinc doped glasses did not promote cell growth when tested against T24 cells.

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Chapter 6

Conclusion and Further work

6.1 Conclusion

The aim of the current research was to develop novel antimicrobial bioactive glasses to prevent catheter associated urinary tract infections (UTI). UTIs affect significant proportions of the population, being particularly prevalent in older adults. Up to 40 % of healthcare associated infections are UTIs and 80% of those are associated with catheter use [1]. Studies have shown that about 15-25% of the patients admitted in hospital require urinary catheterisation [2] which increases their risk of developing urinary tract infection by 5% per day [3, 4]. The financial implications are enormous as the high complication rate arising from catheterisation requires significant time and cost. More than a million cases are reported each year accounting for annual economic burden of approximately £125 million [4, 5]. UTIs are predicted to increase dramatically in the future in line with the rise in average life expectancy. The current study focused on incorporating antibacterial metal ions (Co, Cu and Zn); alone and in combination within a phosphate based glass (P_2O_5 - Na_2O - CaO) to prevent CAUTI's.

The objectives of the chapter 2 were to manufacture and study the dissolution behaviour and related aspects such as pH and ion release profile of un-doped and cobalt or zinc doped phosphate glasses. The results can be summarised as follows:

1. The incorporation of metal oxides (cobalt and zinc oxide), alters the dissolution behaviour of the doped glasses compared to un-doped phosphate based glass. The rate of dissolution of cobalt doped glasses was shown to decrease with an increasing cobalt oxide content whereas for zinc doped phosphate glasses, an increase in dissolution rate was seen with an increasing zinc oxide content. This is due to the charge to size ratio of the dopant metal oxide replacing CaO content [6, 7]. When cobalt content is increased, the weaker $Ca-O$ bonds are replaced by stronger $Co-O$ bonds, making the bond cleavage difficult thus the dissolution decreases with an increasing cobalt oxide

content. Whereas, the increase in dissolution of zinc doped glasses with an increasing zinc oxide content is due to opening of network which makes it easier to ingress water and thus more solubility. Further studies on structural changes occurring due to dopants could strengthen the understanding of the changes in glass dissolution behaviour.

2. A variation in glass dissolution rate was observed in different media, with a decreased dissolution in nutrient broth and cell culture media and an increased solubility in synthetic urine. Various biological media vary greatly in composition, as the ionic strength of the media plays a considerable role in glass dissolution, hence the variation in glass degradation in different media. Significantly high dissolution rate in synthetic urine is likely due to its high pH as the dissolution increases dramatically in highly acidic or alkaline pH. This has also been demonstrated in studies by Bunker *et al*, [8] and Gao 2004 *et al*. [9], where authors demonstrated a strong relationship between pH and glass dissolution.
3. The ion release and pH results of both cobalt and zinc doped glasses are concurrent with the weight loss data. Increase in the release rate of anion and cations was observed in the most soluble compositions compared to less soluble glasses.

The objectives of the chapter 3 were to evaluate the antimicrobial efficacy of cobalt or zinc doped (1, 3, 5 and 10 mol %) phosphate based glasses by testing the surface as well as the dissolution products in aqueous media. The results are summarised below:

1. A strong antimicrobial effect was demonstrated by cobalt and zinc doped phosphate glass surfaces against *E. coli*, *S. aureus* and *C. albicans* compared to un-doped phosphate and control specimens. The antimicrobial effect was time dependent as a

gradual decrease in microbial viability was observed over a period. Furthermore, a concentration dependent effect was also exhibited in the initial hours, as enhanced killing was observed for higher mol % glasses, however the effect plateaued after 24 hours.

2. The dissolution products of cobalt doped phosphate based glasses failed to exhibit bacteriostatic/bactericidal effect against any of the test strains, however dissolution products of 5 and 10 mol % zinc doped glasses showed a kill at 120 hours against *E. coli*. Likewise, significant difference in the activity was seen against *S. aureus* within 48 hours which improved by 120 hours. *C. albicans* on the other hand, showed an antimicrobial effect within 24 hours which appeared to diminish as the time progressed. The dissolution products therefore showed minimum or no activity compared to direct contact experiments. The lack of activity was attributed to the fact that media such as nutrient broth provides a complex environment with many ionic species that may reduce the concentration of free metal ions by precipitation or by the formation of soluble complexes [10, 11]. This could be further investigated by performing inductively coupled plasma optical emission spectrometry (ICP-OES).
3. Whilst an antimicrobial effect was seen against the three strains studied, *E. coli* showed higher susceptibility compared to *S. aureus* and *C. albicans*. As discussed earlier, the cell wall is a primary target in contact mediated killing therefore, the difference in the efficacy against each strain could be due to the difference in outer cell wall. The thick peptidoglycan in *S. aureus* not only confers stability but also has charged anionic clusters which contribute to the binding of ions such as sodium, calcium, copper and cobalt thus promote bacterial adhesion to the metallic surfaces. Whereas in *E. coli* the presence of outer membrane with porins allows passage of

metallic ions which subsequently bring about cell death by inhibiting DNA and proteins.

4. Several studies have been conducted to understand the antimicrobial mode of action of bio-glasses and pH has been shown to play a key role. The pH analysis exhibited that nutrients broth has sufficient capacity to neutralise the minimal changes occurring in surrounding environment due to glass leaching. Therefore, in the present analysis, it can be argued that the antimicrobial effect seen is due to the doped metallic ions, cobalt or zinc.

The emergence of resistant strains and decline in discovery of new antibiotics has led to the idea of combining various antimicrobials to treat resistant strains and/or polymicrobial infections. The study presented in chapter 4 was therefore undertaken to investigate the effect of combining two metal oxide i.e. to determine if the bioactive glasses can be combined to achieve synergistic antimicrobial effect. The results are summarised below:

1. It was shown that 5 mol % cobalt, copper or zinc doped phosphate glass systems possess antibacterial and antifungal activity proving effective against *E. coli*, *S. aureus* and *C. albicans* in planktonic form. The un-doped phosphate based glass did not possess an antimicrobial activity which shows antimicrobial efficacy of the doped glasses is derived from the cobalt, copper or zinc ions.
2. The present study has established that metal doped bioactive glasses can be used in combination as they possess a synergistic activity. A synergistic antimicrobial activity was observed for Cu/Co and Cu/Zn against *E. coli* and Cu/Zn against *S. aureus*.

The aim of the chapter 5 was to study the cytotoxicity of the given glasses (3, 5 and 10 mol % cobalt or zinc doped phosphate glasses) against human cells as biocompatibility of bioactive glasses is significant when considering their *in vivo* applications. Therefore, direct and indirect exposure of glasses to primary Human Uroepithelial Cells and T24 bladder cancer cells was investigated. The results were as follows:

1. The effect of dissolution products of un-doped phosphate glass and the direct contact assay shows reduced viability of HUCs and T24 after 24 and 48 hours respectively. Dissolution products of cobalt doped glasses demonstrated a strong cytotoxic effect against normal HUCs, in a concentration dependent manner. Due to a difference in the pH of McCoy's media, pH buffered and non-buffered analysis was undertaken. Apart from the dissolution products of 3 mol % cobalt doped glass (pH buffered), the viability of T24 cells was compromised in a dose dependent manner in both pH buffered and non-buffered analysis. Thus, a reduced cell viability of both cell types was demonstrated with dissolution products of cobalt doped glasses where 10 mol % glass was the most toxic composition.
2. Like the dissolution products of cobalt doped glasses, zinc doped phosphate based glasses also showed a significant cytotoxic effect on primary human uroepithelial cells. However, a proliferative effect was observed against T24 cells. The decrease in cell viability could be attributed to the ionic strength of the conditioned media. Bioactive glasses when immersed in an aqueous media degrade and release ionic products, which if higher than the optimum range are detrimental to cells.

3. The direct contact experiments were only conducted against T24 cells. The results from direct contact experiment show that cobalt or zinc doped glasses did not promote cell growth when tested against T24 cells. The significant cytotoxicity is attributed to the fact that the experiments were conducted in a 6 wells plate, which can accommodate a maximum of 2 ml of cell culture media. The small quantity of media means an increased ionic strength and considerable changes in the pH which proved detrimental to cells. Therefore, further investigation in larger vessel such as T25 or T75 flask could provide a better insight into the effect of direct contact of uroepithelial cells with the given bioactive glasses.

The given study demonstrates that doping phosphate glass with cobalt, copper, zinc or their combinations confer antimicrobial properties to the glass system. However, the antimicrobial properties of the glasses are significantly modulated by the glass degradation behaviour in aqueous media. Whilst *in vitro* studies undertaken here are useful to assess the antimicrobial and cytotoxic behaviour of the glasses, it is difficult to generalise the results to the *in vivo* behaviour of the glasses. A biological fluid such as urine is a complex environment which contains constituents that broth or cell culture media lack, therefore the dissolution and ion leaching of the glasses is likely to vary and thus their antimicrobial and cytotoxic effect.

Nevertheless, even though the glasses, due to their cytotoxicity, cannot be used inside the bladder they can still be used to prevent the catheter associated infections. As mentioned in section 1.4.2.5, 34% microorganisms enter the urinary tract due to intraluminal migration associated with manipulation of collecting systems or due to contamination of the drainage bag or disruption of the catheter tubing junction, therefore the preventing microorganisms from this route can reduce the infection rate. This can be achieved by introducing a cartridge containing with 5 mol % cobalt or copper doped phosphate glass individually or glass doped with combination of 5 mol % cobalt,

copper or zinc in collecting bag which can dissolve slowly over a period and thus prevent microbial adhesion.

Since the cell study has demonstrated a concentration dependent toxic effect the glasses, therefore coating a catheter (internally and externally) with a decreasing gradient of cobalt or zinc doped phosphate glasses is likely to prevent the occurrence of infection with minimal cytotoxicity. This can be achieved by coating the distal part of the catheter with the highest possible mol % of the doped glass and decreasing the concentration towards the drainage port (the part inserted inside the bladder). The use of phosphate based glasses as a delivery system will not only provide a controlled sustained release of antimicrobial ions but also will have additional benefit of low pH. The acidic pH will prevent formation of calcium and magnesium crystals which eventually will minimize the risk of catheter encrustation and blockage.

6.2 Further work

Whilst this thesis has shown the potential use of phosphate based glasses doped with divalent metallic ions for preventing catheter associated urinary tract infections, nonetheless certain limitations remain. The given section explores the limitations and opportunities for extending the scope of this thesis.

1. The weight loss data (section 2.4.1.2 and 2.4.2.2) was conducted in four different media; distilled water, nutrient broth, cell culture media and synthetic urine. As discussed previously in section 2.5, the pH of the media plays a key role in determining the rate of glass dissolution. Since the initial pH of Surine TM was highly alkaline therefore a considerably higher dissolution rate (by a factor of seven) was observed in comparison to the other aqueous media tested. Therefore, it is highly desirable to repeat the dissolution rate in simulated urine that is more representative of the physiological conditions more specifically within an optimum pH range (5.5 - 7.0).

2. An exceptional trend in the dissolution behaviour of zinc doped phosphate glasses was observed. As discussed in section 2. 5, glass dissolution is governed by the glass structural therefore a detailed structural analysis of zinc doped phosphate glasses is required to capture the changes in the glass network that affect the dissolution of zinc doped glasses. Since zinc behaves as an intermediate oxide and can act as network former or modifier therefore it is required to understand the role of zinc oxide in the present glass system. This can be achieved by performing neutron diffraction analysis along with X-ray absorption spectroscopy to determine the coordination environment of zinc with other elements. This will provide an insight into whether zinc act as the network former and/or modifier in the glass system studied.
3. The antimicrobial studies were performed in standard laboratory broths and media which does not essentially mirror the urinary environment, therefore it would be ideal to perform antimicrobial assays in artificial urine that is capable of growing urinary pathogens. A simple artificial urine recipe for growth of urinary pathogen can be found in the reference [12].
4. The findings of cell studies showed a proliferative effect of the dissolution products of zinc doped phosphate glasses against T24 cell, however a significant cytotoxic effect was observed on the viability of HUCs. As proposed earlier this is likely due to the presence albumin in the media used to grow T24 cells. It has been reported [13-15] that albumin forms soluble complexes with zinc ions, therefore once bound to albumin zinc ions are not available and thus the lack of cytotoxic effect was seen. Therefore it is desirable to quantify the free ions in different media to support the above statement. This could be achieved by performing elemental analysis i.e. ICP-OES.

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Appendix

Determination of antimicrobial efficacy of six pure metals against *Escherichia coli* and *Staphylococcus aureus*

A pilot study was conducted to determine antimicrobial efficacy of pure metals; silver, copper, cobalt, zinc, nickel and gallium at the given concentrations of 2.5, 5.0, 10.0 and 20.0% w/v against Gram negative (*Escherichia coli* NCTC 10538) and Gram positive bacteria (*Staphylococcus aureus* ATCC 6538).

Method: 50 mg, 100 mg, 200 mg and 400 mg of each metal powder was added in 2 ml of 10^6 CFU/ml bacterial culture to make 2.5%, 5%, 10% and 20% w/v solutions respectively. Each sample along with a control was incubated for a period at 37°C in a shaking incubator with a shaking speed of 100 rpm. Following incubation for 24, 48, 72 and 96 hours, tenfold dilutions were prepared for each sample. 10^{-2} , 10^{-4} and 10^{-6} dilutions were plated in duplicates on to nutrient agar to estimate viable count. The reduction in bacterial density was measured as reduction in log CFU/ml.

Results:

Figure A.1 shows reduction in log CFU/ml for *E. coli* when incubated with the metal powders for 24 hours. 5% copper shows a considerable decrease, 4 log reductions, in bacterial cell density compared to the control whereas, 10% copper shows almost seven logs reduction. 2.5% and 5.0% cobalt both demonstrated 4 logs reduction and 10% cobalt showed a 9-log reduction compared to the control. Nickel showed a trivial reduction in bacterial number following 24 hours exposure. Gallium and silver the number of bacteria raised to a level above enumeration.

The viable counts of *S. aureus* following direct contact exposure, for 24 hours, are shown in Figure A.2. Except for gallium and silver all metals showed antimicrobial effects. The bacterial numbers showed a considerable decrease within 24 hours for copper, cobalt and zinc compared to the control. 10% copper and cobalt showed a seven-log reduction i.e. complete kill compared

to the control. Whereas the growth of microorganism was very high for gallium and silver samples and reached a level above enumeration limit.

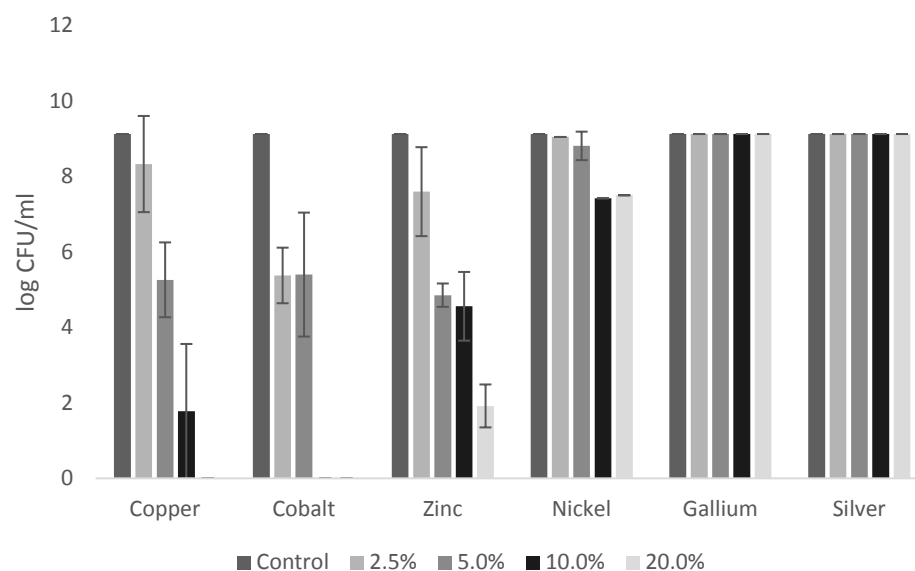


Figure A.1: showing antimicrobial action of Cu, Co, Zn, Ni, Ga and Ag against *E. coli* following 24 hours exposure.

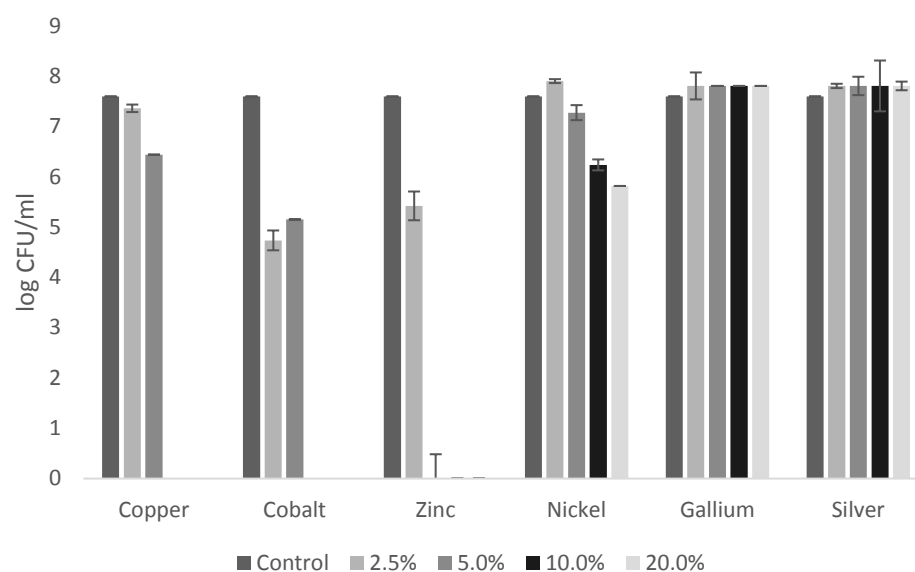


Figure A.2: showing antimicrobial action of Cu, Co, Zn, Ni, Ga and Ag against *S. aureus* following 24 hours exposure.

Metal powders that did not show any considerable antimicrobial effect in 24 hours; nickel, silver, gallium (against *E. coli* and *S. aureus*) and zinc (against *E. coli*), were studied for longer hours. The results of long interval sampling showed a considerable reduction in bacterial number for nickel and zinc. Antimicrobial effect of metals when observed against *E. coli* (Figure A.3), showed a rapid decrease in bacterial number by zinc and nickel within 48 hours. A significant decrease, 7 logs reduction, was observed for zinc within 24 hours and a complete kill was seen in the next 24 hours compared to the control. While nickel did not show any antibacterial effect in 24 hours; a complete kill, 8 logs reduction, was observed by the end of 48 hours. Figure A.3 illustrates a 2 logs reduction in bacterial density compared to the control in case of silver however no noticeable decrease was observed for gallium.

According to Figure A.4, showing antimicrobial effect against *S. aureus*, nickel showed a gradual decrease in bacterial density over 96 hours compared to the control. By the end of the assay period a complete kill was observed which was approximately six logs less. When incubated for longer hours, silver and gallium did not show any bacterial reduction compared to the control or original number of bacteria.

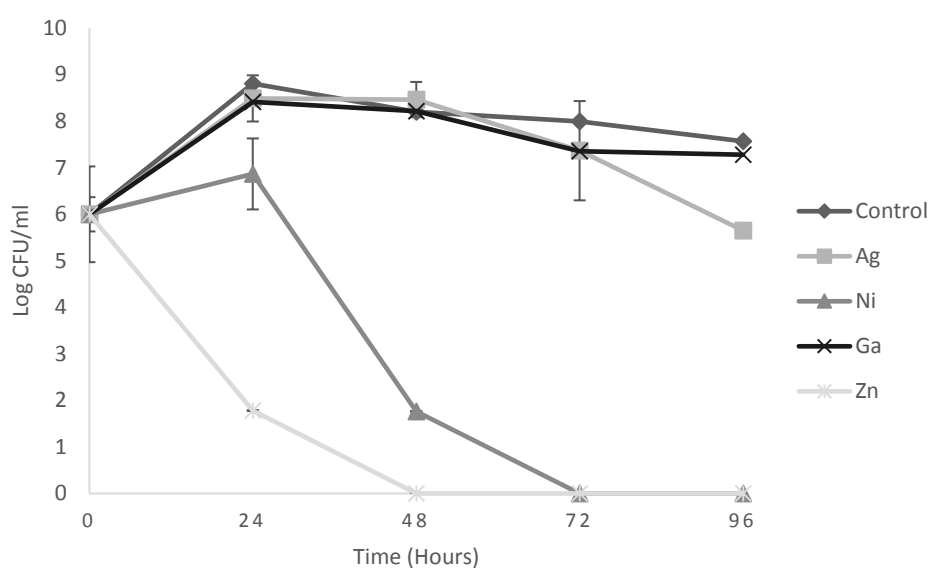


Figure A.3: showing antimicrobial action of Zn, Ni, Ga and Ag against *E. coli* following 96 hours exposure to metals.

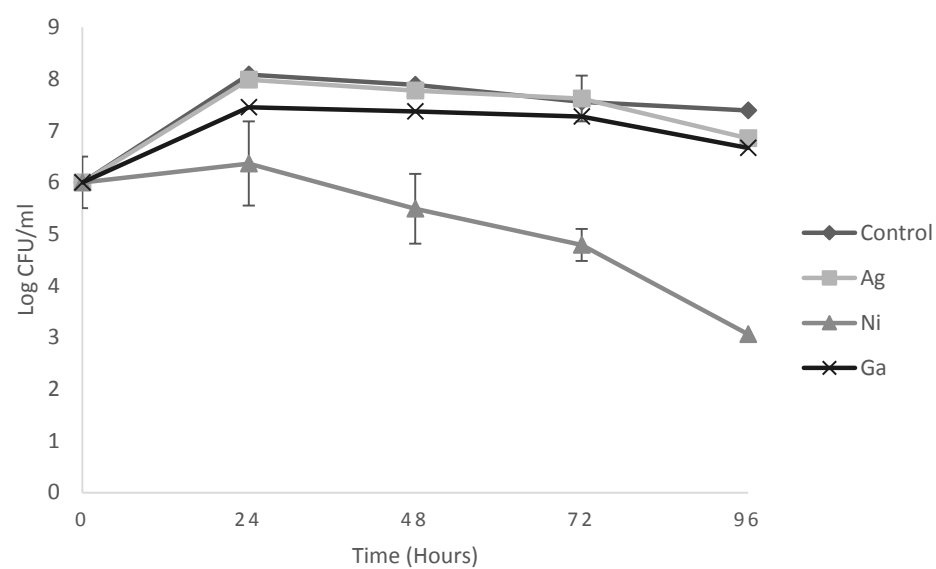


Figure A.4: showing antimicrobial action of Zn, Ni, Ga and Ag against *S. aureus* following 96 hours exposure to metals.

Standard curve for colony forming units using optical density

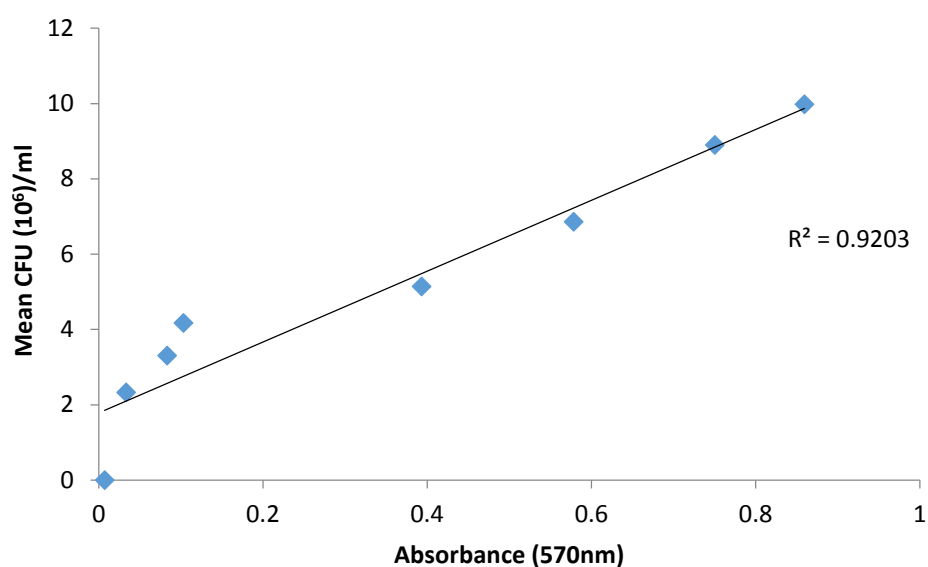


Figure A.5: Mean colony forming units of *E. coli* (NCTC 10538) correlated to turbidity measured at an absorbance of 570 nm.

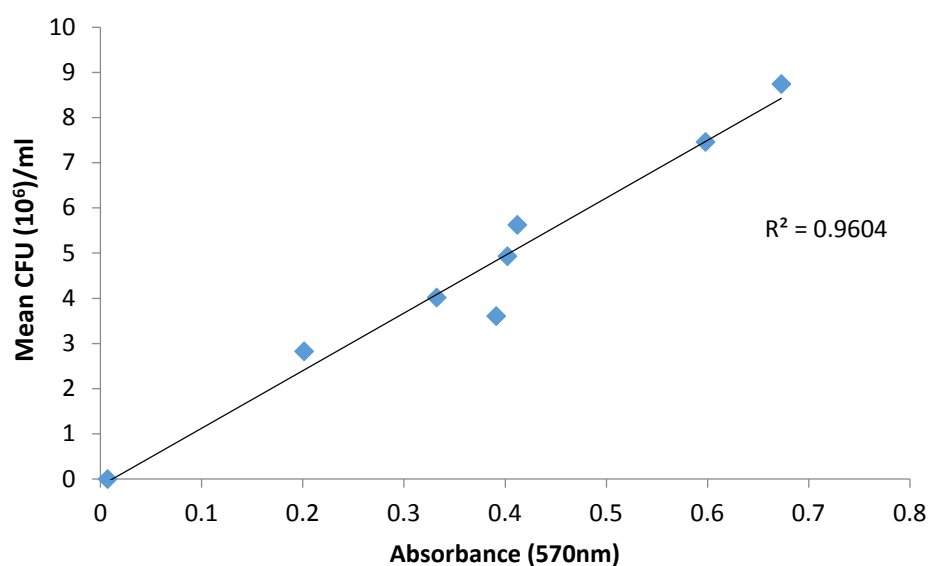


Figure A.6: Mean colony forming units of *S. aureus* (ATCC 6538) correlated to turbidity measured at an absorbance of 570 nm.

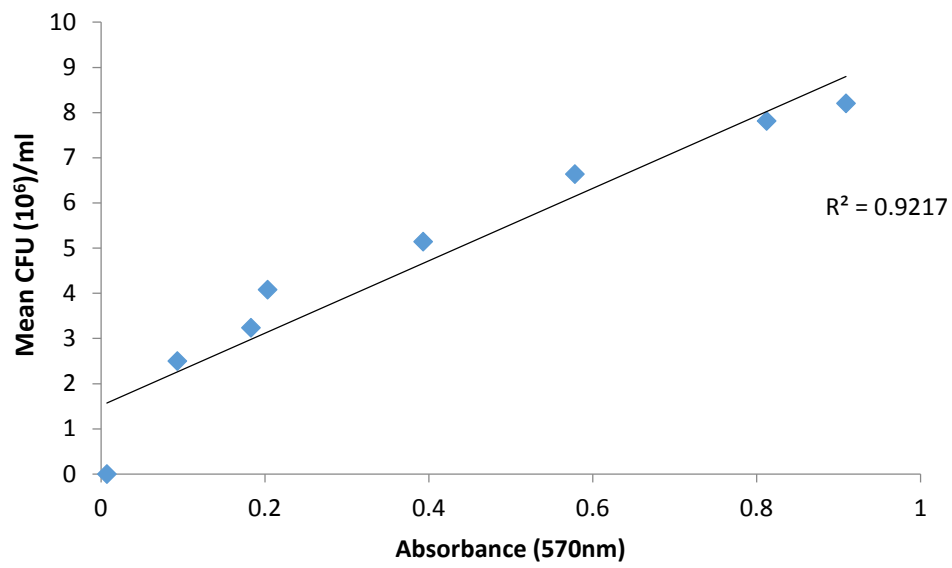


Figure A.7: Mean colony forming units of *C. albicans* (ATCC 76615) correlated to turbidity measured at an absorbance of 570 nm.

Glass composition	0	2 hour	6 hour	24 hour
0%	7.03±0.11	6.79±0.14	6.18±0.12	6.04±0.21
5% Co	7.03±0.11	6.73±0.17	6.34±0.45	5.96±0.08
5% Cu	7.03±0.11	6.52±0.12	6.0±0.01	4.79±0.16
5% Zn	7.03±0.11	6.39±0.12	6.04±0.11	5.26±0.01
Co/Cu	7.03±0.11	6.38±0.39	6.12±0.37	4.83±0.14
Co/Zn	7.03±0.11	6.45±0.34	6.18±0.42	5.93±0.08
Cu/Zn	7.03±0.11	6.23±0.20	5.98±0.11	4.83±0.25

Table A.1: The pH readings of broth taken at 0, 2, 6, and 24 hours incubation with glass particles at 37°C and 200 rpm.

Glass composition	pH
0%	7.59
3% Cobalt	7.62
5% Cobalt	7.61
10% Cobalt	7.67
3% Zinc	7.71
5% Zinc	7.73
10% Zinc	7.58

Table A.2: The pH readings of conditioned media taken after 24 hours incubation with discs at 37°C and 200 rpm in Urothelial cell media (UCM).

Glass composition	Before pH buffering	After pH buffering
0%	8.22	7.60
3% Cobalt	8.24	7.58
5% Cobalt	8.32	7.56
10% Cobalt	8.25	7.55
3% Zinc	8.14	7.70
5% Zinc	8.33	7.68
10% Zinc	8.24	7.69

Table A.2 shows the pH readings of conditioned media taken after 24 hours incubation with discs at 37°C and 200 rpm in McCoy's 5A media. pH readings were also taken after media acclimatization in 5% CO₂ incubator.

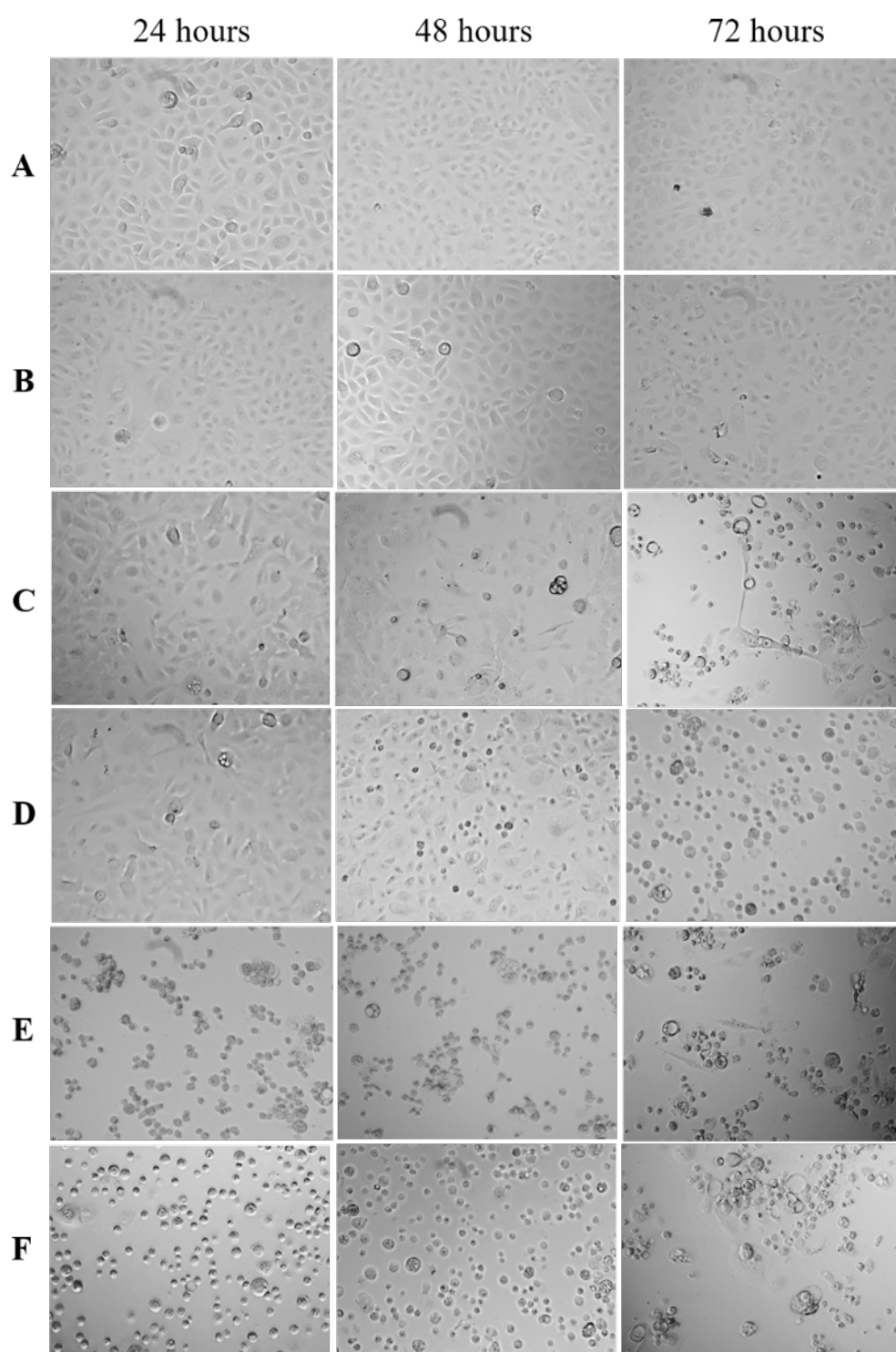


Figure A.8: Phase contrast images of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of cobalt doped phosphate based glasses. Cells grown in **(A)** complete media, media conditioned with **(B)** un-doped phosphate glass, **(C)** 3% cobalt glass, **(D)** 5% cobalt glass **(E)** 10% cobalt glass and **(F)** cells treated with 70% (v/v) ethanol. Images were taken at 100x magnification.

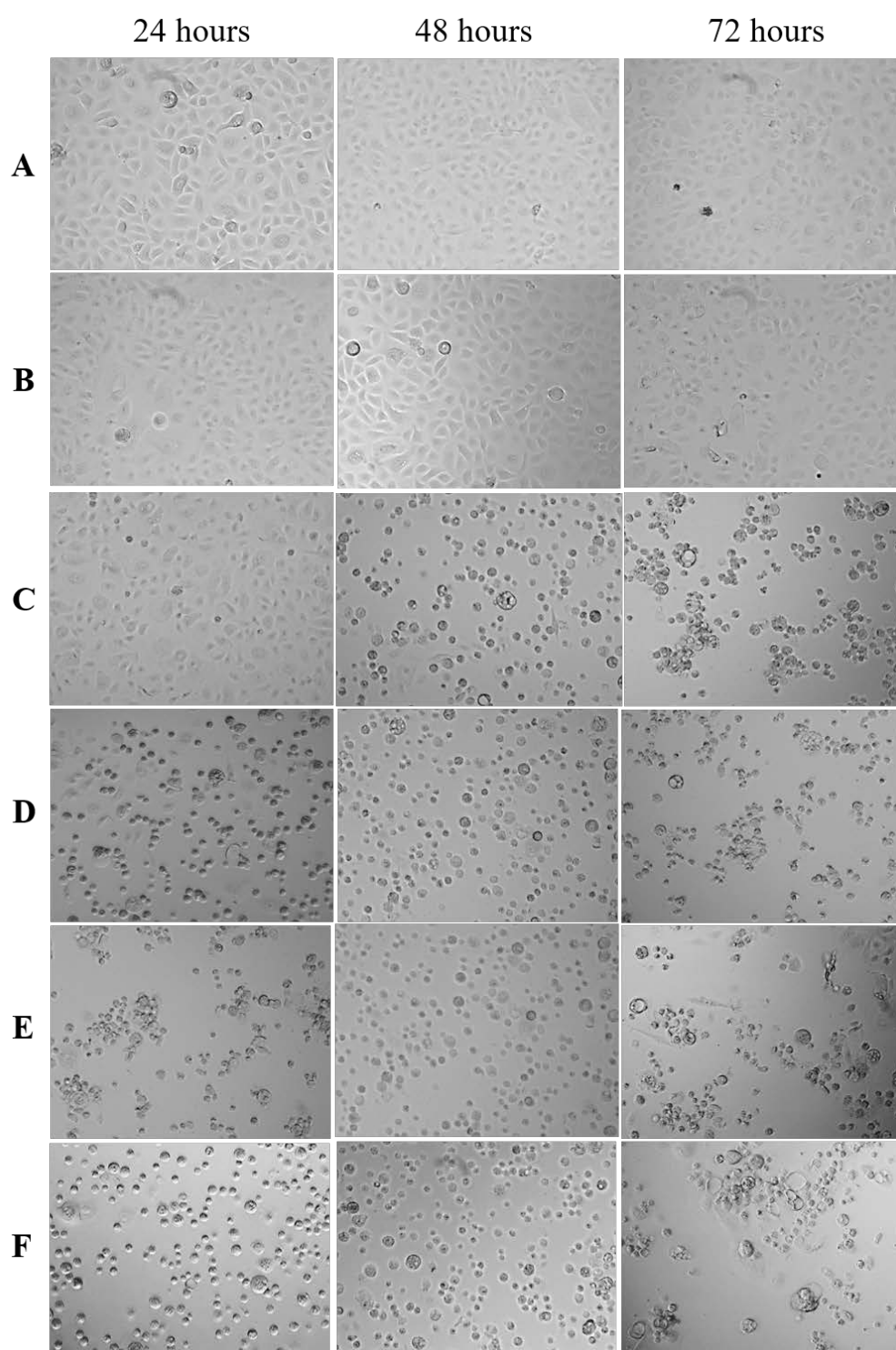


Figure A.9: Phase contrast images of Human uroepithelial cells cultured for 24, 48 and 72 hours in dissolution products of cobalt doped phosphate based glasses. Cells grown in (A) complete media, media conditioned with (B) un-doped phosphate glass, (C) 3% zinc glass, (D) 5% zinc glass (E) 10% zinc glass and (F) cells treated with 70% (v/v) ethanol. Images were taken at 100x magnification.

Presentations and Professional activities

Oral Presentations

Raja F. The antimicrobial efficacy of cobalt and zinc doped phosphate based glasses (2016)
Society of Glass Technology, Centenary Conference, Sheffield, UK.

Raja F. Development and characterisation of cobalt doped bioactive glasses (2016). Life and
Health Sciences Postgraduate Research Day, Aston University, Birmingham, U.K.

Raja F. Prevention of catheter associated urinary tract infections with bioactive glasses (2015).
Research in Progress Seminar, Aston University, Birmingham, U.K.

Raja F. Strategies for prevention of catheter associated urinary tract infections (2014). Research
in Progress Seminar, Aston University, Birmingham, U.K.

Poster Presentations

Raja F, Martin R and T. Worthington. (2016) The antimicrobial efficacy of metallic copper,
cobalt, zinc and silver nanoparticles European Congress of Clinical Microbiology and Infectious
Diseases, Amsterdam.

Raja F, Martin R and T. Worthington. (2016) The antimicrobial efficacy of metallic copper,
cobalt, zinc and silver nanoparticles Federation of Infectious Societies, Glasgow, UK.

Raja F, T. Worthington and Martin R. (2015) Investigation of antimicrobial efficacy of pure metal
nanoparticles against Gram positive and Gram negative bacteria. Aston University Postgraduate
Poster Day, Aston University, Birmingham, U.K.

Raja F, T. Worthington and Martin R. (2014) Strategies for prevention of catheter associated
urinary tract infections in elderly. Aston University Postgraduate Poster Day, Aston University,
Birmingham, U.K.

Raja F, T. Worthington and Martin R. (2014) Strategies for prevention of catheter associated urinary tract infections in elderly. ARCHA Symposium, Aston University, Birmingham, U.K.

Raja F, T. Worthington and Martin R. (2014) Strategies for prevention of catheter associated urinary tract infections in elderly. British Society for Research on Ageing, Liverpool, U.K.

Related Professional activities

June 2016 and 2014 – Participated in organising and running Healthcare aspiration event at Aston University, Birmingham, UK.

November 2015 – Delivered talks to elderly people on Bioactive glasses in ARCHA event

2013 - 2014 Delivered and helped in organising Microbiology roadshows in schools across Birmingham and Walsall.

Oct 2014 - Mar 2017: Designed and supervised undergraduate dissertation projects.

Publications

The antimicrobial efficacy of cobalt doped phosphate based glass against Gram positive and Gram negative bacteria

The release of antimicrobial zinc ions from phosphate based glass for treating catheter associated urinary tract infections.

The antimicrobial efficacy of pure copper, cobalt, zinc and silver nanoparticles: alone and in combination